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Volume 13 (2); June 25, 2023

Review

Newcastle Disease Virus in Poultry: Current Status and Control Prospects

Moustapha A, Talaki E, Akourki A, and Ousseini M.

World Vet. J. 13(2): 240-249, 2023; pii:S232245682300026-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj26

ABSTRACT: Since its first appearance in Java Island, Indonesia, in 1926, Newcastle disease has significantly impacted the global poultry industry, leading to substantial economic losses. The disease has rapidly spread worldwide, becoming endemic in many countries where agriculture is the primary source of national income. The present study aimed to present a comprehensive review of the recent literature on the Newcastle disease virus to contribute to understanding the virus and its control measures in poultry and provide an updated perspective on current knowledge. All strains of the Newcastle disease virus are classified under a single serotype; however, they are grouped into two classes and have been found to have emerging genetic diversity. Although various molecular diagnostic protocols have been developed, many have limitations. Nucleotide variability in the F gene of the Newcastle disease virus seems to explain the false-negative results provided by different real-time reverse transcription polymerase chain reaction protocols. Vaccination combined with biosecurity measures has been shown to limit the devastating effect of the Newcastle disease virus. However, the current vaccines are not effective enough to prevent viral shedding and infection of vaccinated animals. The efficacy of the vaccine strains utilized for decades is being scrutinized, raising questions about their effectiveness over time. The development of reverse genetics offers promising prospects for exploring new generations of attenuated vaccines capable of protecting poultry against clinical diseases and infections, such as Newcastle disease.
Keywords: Diagnosis, Genotype, Newcastle disease, Pathogenicity, Poultry, Vaccination

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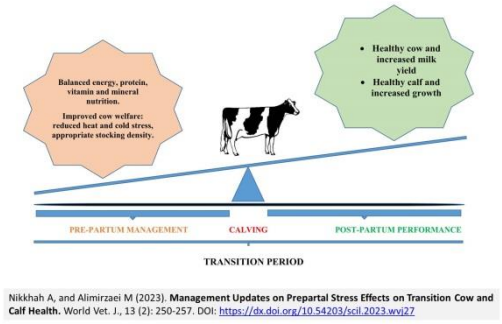
Review

Management Updates on Prepartal Stress Effects on Transition Cow and Calf Health

Nikkhah A, and Alimirzaei M.

World Vet. J. 13(2): 250-257, 2023; pii:S232245682300027-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj27



ABSTRACT: The transition phase is thought to be the most critical period in high-producing dairy cows' productive cycle. Maternal stresses during the peripartum period affect both dam and newborn calf health. The objective of this review article was to describe the role of prepartal metabolic and environmental stressors on postpartum-related disorders in both cows and newborn calves. The transition phase (21 d before to 21 d after calving) is considered the most critical period in dairy cattle life. Decreased dry matter intake coincides with increased nutrient demands due to the onset of lactation. It can lead to negative energy balance and tremendous metabolic challenges for high-producing dairy cows. During this time, insulin concentrations and peripheral tissue sensitivity decrease, leading to fat mobilization from adipose tissue. Such incidences would increase levels of non-esterified fatty acids and beta-hydroxybutyric acid in plasma. The success in the transition from the dry to lactating phase depends on how the cow could deal with such challenges. Failure to handle such metabolic alterations may predispose cattle to costly metabolic disorders such as ketosis, fatty liver, retained placenta, displacement abomasum, and infectious diseases, including metritis and mastitis in the postpartum period. The energy content of dairy cow diets in the dry-off (40 days before calving) or close-up (21 days before calving) periods may influence cow physiology and health in the peripartum period. The role of a transition period diet on cow health and productivity in the early or overall lactation period has been frequently investigated. However, the role of stressors such as nutritional deficiencies, heat stress, stocking density, and grouping in the late gestation period on the health and performance of cows and calves are much less addressed. Therefore, the present review delineates and reviews updates

on the role of maternal stresses during the peripartum period on dam as well as newborn calf physiology and behavior.
Keywords: Immunity, Management, Metabolic disease, Prepartal stress, Transition cow

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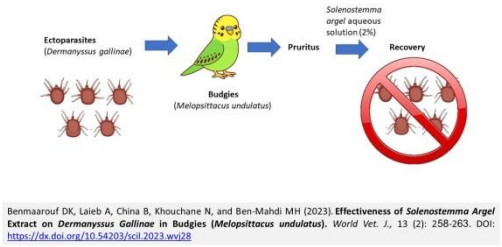
Research Paper

Effectiveness of Solenostemma Argel Extract on Dermanyssus Gallinae in Budgies (*Melopsittacus undulatus*)

Benmaarouf DK, Laieb A, China B, Khouchane N, and Ben-Mahdi MH.

World Vet. J. 13(2): 258-263, 2023; pii:S232245682300028-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj28



Benmaarouf DK, Laieb A, China B, Khouchane N, and Ben-Mahdi MH (2023). **Effectiveness of *Solenostemma Argel* Extract on *Dermanyssus Gallinae* in Budgies (*Melopsittacus undulatus*).** *World Vet. J.*, 13 (2): 258-263. DOI: https://dx.doi.org/10.54203/scil.2023.wvj28

ABSTRACT: *Dermanyssus gallinae* (*D. gallinae*) is an important ectoparasite in veterinary and human medicine due to its role as a vector of infectious disease-causing pathogens and its economic impact. The present study reported the infestation of 45 budgies (*Melopsittacus undulatus)* reared in an aviary at the Jardin d'essai Zoo, Algiers, Algeria, showing signs of pruritus with sores due to itching. Skin samples were taken from the 45 budgies (26 females, 19 males), 26 nests, and an aviary containing the budgies were microscopically analyzed. The findings indicated the presence of *D. gallinae (*hematophagous mite*)* in all budgies and nests. To fight against this red mite, a treatment based on the *Solenostemma argel* (*S. argel*) aqueous extract was implemented. The *S. argel* aqueous extract 2% showed a fast and effective influence on *D. gallinae* mites present in the budgies, nests, and aviary. The aqueous extract of *S. argel* leaves could be a good candidate in the fight against red mites.
Keywords: Aqueous extract, Budgie, *Dermanyssus gallinae*, *Solenostemma Argel*

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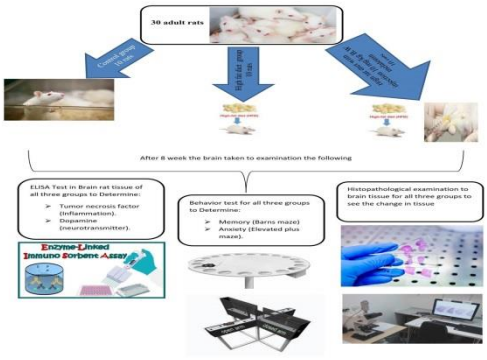
Research Paper

The Protective Effects of Melatonin against Brain Disorders Induced by the Western Diet in Male Rats

Rayshan AR, Abdulwahid AA, and Alsaedi AA.

World Vet. J. 13(2): 264-284, 2023; pii:S232245682300029-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj29



ABSTRACT: Globally, the effects of consuming a diet rich in fat have gained great concerted attention. The current study was conducted to evaluate the protective effects of melatonin on neurological disorders induced by the western diet in rats. A total of 30 adult male white local Iraqi rats were randomly assigned to three equal groups, including control (CC), high-fat diet (HFD), and melatonin group (HFD+M, a high-fat diet along with intraperitoneal injections of 10 mg/kg body weight melatonin) for 8 weeks. The rats were analyzed in terms of brain tissue concentration of dopamine, tumor necrosis factor (TNF), and nervous system impairment using Barns maze task and elevated plus maze. The findings revealed a significant decline in the dopamine concentration of the HFD group after 8 weeks of treatment, compared to CC and HFD+M groups. Moreover, there was a significant increase in brain TNF-α concentration in the group fed HFD, compared with CC and HFD+M. Finally, the melatonin treatment significantly reduced spatial memory impairments and anxiety induced by HFD in rats. After 8 weeks, the histological examination revealed that brain section rats on an HFD indicated significant congestion in the blood vessels with marked cerebral edema, where there was a dilation of Virchow-Robin space, severe congestion, and infiltration of inflammatory cells in the meninges. The HFD+M groups showed normal meninges without any inflammatory exudate except for a few congestions in the blood vessels and no or mild vacuolations in the cerebral tissue, gliosis, and astrocytosis. In contrast, male rats fed an HFD showed vacuolation and aerophagia in brain tissue and a marked aggregation of the proliferation of astrocytes and a proliferation of microglial cells in the cerebral. In conclusion, HFD impairs brain neurotransmitters, induces pro-inflammatory changes, and affects learning ability and memory by changing the structure of neural tissue. Melatonin can ameliorate HFD-caused effects.
Keywords: Brain, High-fat diet, Inflammatory cells, Learning ability, Melatonin, Nervous system

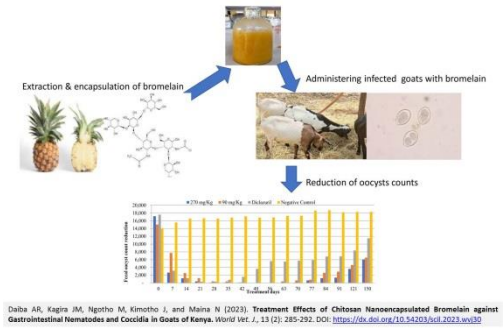
[Full text-PDF] [Crossref Metadata] [Scopus] [Export from ePrints]

Research Paper

Treatment Effects of Chitosan Nanoencapsulated Bromelain against Gastrointestinal Nematodes and Coccidia in Goats of Kenya

Daiba AR, Kagira JM, Ngotho M, Kimotho J, and Maina N.

World Vet. J. 13(2): 285-292, 2023; pii:S232245682300030-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj30



ABSTRACT: The management of gastrointestinal nematodes (GIN) and coccidiosis of livestock relies on the use of commercial anthelmintic; however, the excessive and frequent usage of these drugs has led to the substantial and dramatic development of anthelmintic and anticoccidial resistance. The present study aimed to evaluate the anthelmintic and anticoccidial efficacy of chitosan nanoencapsulated bromelain (CNB) against a wide spectrum of GIN and coccidia in goats. Additionally, the study assessed the safety of CNB in the goats. Bromelain was extracted from the pineapple peels and then encapsulated using chitosan. A total of 20 healthy male goats naturally infected with GIN and coccidia were used. The goats were separated into four treatment groups, with five goats per each. The CNB was orally administered at dosages of 270 and 90 mg/Kg, once daily for 60 days. Fecal egg counts (FEC), fecal oocyst counts (FOC), packed cell volume (PCV), aspartate aminotransferases (AST), alanine aminotransferases (ALT), urea, and creatinine were determined weekly. The goats were monitored for clinical signs daily, and their body weight was recorded weekly. The findings revealed that FEC reduction rates in the group that received 270 mg/Kg CNB and the group that received Albendazole were 73.41% and 79.54% at day 7 post-treatment. Also, the reduction of FOC in the group receiving 270 mg/Kg CNB at day 7 (84.12%) did not show a significant difference with Diclazuril (82.12%). The FEC and FOC were zero (reduction of FEC and FOC was 100%) at 28 days of treatment in goats treated with 270 mg/Kg CNB. During the monitoring period, no mortalities and no clinical signs were observed in the treated goats. The PCV, AST, ALT, creatinine, and urea levels for the goats in all groups were within normal limits. No pathological lesions were observed in the goat's organs. In conclusion, the results demonstrated that repeated (60 days) dosages of 270 mg/Kg had anthelmintic and anticoccidial effects and were safe for goats. The study recommends further investigation in a field setting involving more animals. This would allow the development of a novel product for managing helminthiasis and coccidiosis in ruminants.
Keywords: Anthelmintic, Anticoccidial efficacy, Bromelain, Chitosan, Encapsulation, Goat

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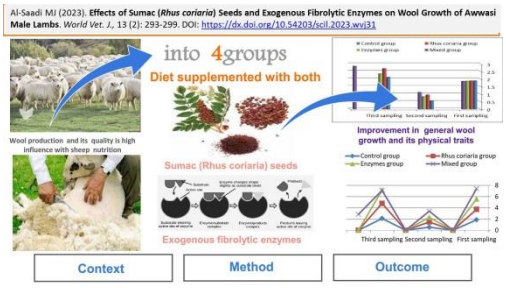
Research Paper

Effects of Sumac (*Rhus coriaria*) Seeds and Exogenous Fibrolytic Enzymes on Wool Growth of Awassi Male Lambs

Al-Saadi MJ.

World Vet. J. 13(2): 293-299, 2023; pii:S232245682300031-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj31



ABSTRACT: Nutrition can have a significant effect on animal production. In recent years, many compounds have been widely used as feed additives to stimulate animals' appetites and consequently improve animal productivity. Exogenous fibrinolytic enzymes are one of these feed additives, which have been used as a digestive stimulant in different types of animals. Sumac (*Rhus coriaria*) seeds and leaves have been widely used as an appetite stimulant. Therefore, this study aimed to determine the dietary effects of using 0.3% exogenous fibrolytic enzymes and 3% of grinds *Rhus coriaria* seeds on wool production and some physical traits of the Awassi lambs. Twenty-four male Awassi lambs with an average age of 4 months were randomly assigned to four dietary treatments, each containing six animals. The control group received a basal diet equivalent to 2% of body weight. The second group received the same diet supplemented with 3% sumac (*Rhus coriaria*) powder. The third group received the basal diet supplemented with 0.3% exogenous fibrolytic enzymes (protease, amylase, and cellulase). The fourth group received the basal diet supplemented with both 3% *Rhus Coriaria* powder and 0.3% exogenous fibrolytic enzymes. The experiment lasted 130 days in the animal house belonging to the College of Veterinary Medicine in Iraq. Some wool traits, including wool staple length, clean wool weight, greasy wool weight, wool fiber length, and wool fiber diameter, were measured. The results revealed significant differences in all measured wool quality traits among the treated groups compared to the control group. The group receiving the diet supplemented with a combination of exogenous fibrolytic enzymes and *Rhus coriaria* powder exhibited the most significant improvements in wool growth, overall wool production, and physical characteristics. These findings highlight the potential of using exogenous fibrolytic enzymes and sumac as effective appetite stimulants and enhancers of wool production in Awassi lambs.

Keywords: Awassi Lambs, Fibrolytic enzymes, Staple, *Rhus Coriaria,* Wool

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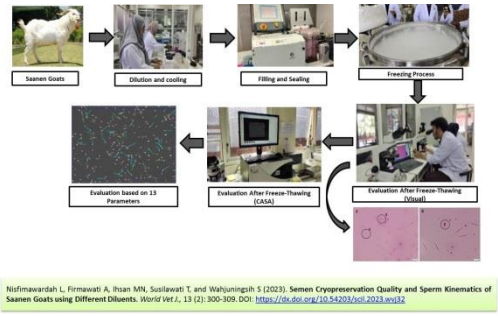
Research Paper

Semen Cryopreservation Quality and Sperm Kinematics of Saanen Goats Using Different Diluents

Nisfimawardah L, Firmawati A, Ihsan MN, Susilawati T, and Wahjuningsih S.
World Vet. J. 13(2): 300-309, 2023; pii:S232245682300032-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj32



Nisfimawardah L, Firmawati A, Ihsan MN, Susilawati T, and Wahjuningsih S (2023). **Semen Cryopreservation Quality and Sperm Kinematics of Saanen Goats using Different Diluents.** *World Vet J.*, 13 (2): 300-309. DOI: https://dx.doi.org/10.54203/scil.2023.wvj32

ABSTRACT: The success of artificial insemination (AI) in small ruminants, especially goats, depends on the quality of frozen semen. Therefore, the current study aimed to determine the quality of various diluents, including tris-egg yolk, AndroMed®, and OviXcell®, on semen quality. The fresh semen samples from three male Saanen goats aged 1.5-2 years were collected and the mean individual motility of samples was recorded at 70%. The cryopreservation quality of the semen was evaluated based on motility, viability, abnormality, and total sperm motility (TSM) indexes. The present laboratory experiment was performed with 3 treatments and 10 repetitions. The treatments in this study were T0 (tris-egg yolk), T1 (AndroMed®), and T2 (OviXcell®). The results showed no significant difference in the parameters of motility, viability, abnormality, and TSM among the treatment group. The kinematic parameters' average path length, velocity curved linear, and linearity showed a significant difference in all treatment groups. However, there were no significant differences among the three groups in terms of motility, progressiveness, distance curved line, distance straight line, average velocity path, velocity straight line, straightness, amplitude lateral head, beat cross frequency, and wobble kinematic parameters. Motility was higher in T2 than in T0 and T1, viability was higher in T1 than in T0 and T2, and abnormality was lower in T1 than in T0 and T2. In conclusion, the use of various diluents, such as tris-egg yolk, AndroMed®, and OviXcell®, can maintain the quality of frozen spermatozoa for over 24 hours, including motility, viability, abnormality, and TSM. Kinematic parameters obtained using CASA IVOS II can provide relevant information for various parameters using these diluents.
Keywords: Computerized Assisted Sperm Analyzer, Goat's sperm, Saanen goats, Semen quality, Sperm cryopreservation

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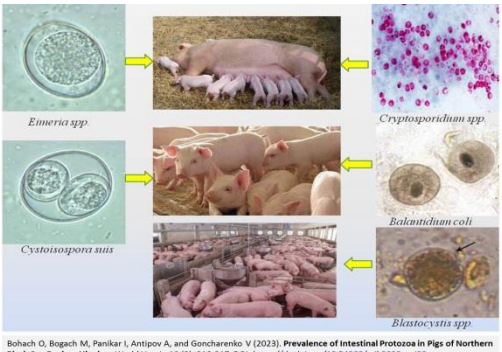
Research Paper

Prevalence of Intestinal Protozoa in Pigs of Northern Black Sea Region, Ukraine

Bohach O, Bogach M, Panikar I, Antipov A, and Goncharenko V.
World Vet. J. 13(2): 310-317, 2023; pii:S232245682300033-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj33



Bohach O, Bogach M, Panikar I, Antipov A, and Goncharenko V (2023). **Prevalence of Intestinal Protozoa in Pigs of Northern Black Sea Region, Ukraine.** World Vet. J., 13 (2): 310-317. DOI: https://dx.doi.org/10.54203/scil.2023.wvj33

ABSTRACT: Intestinal protozoan parasites threaten the health and welfare of pigs and impair the sustainability of pig farms, resulting in monetary losses. The present study aimed to determine the distribution of protozoa in large white pigs in the farms of Odesa, Mykolaiv, and Kherson regions in Ukraine. The parasitological surveys were conducted from March 2020 to March 2022 on three types of farms, including four large farms (> 100 sows), six medium farms (25-100 sows), and eight small farms (< 25 sows). A total of 3938 fecal samples from pigs of various age groups, namely 0-2-month piglets, 2-4-month piglets, pigs on fattening, and sows, were examined. *Eimeria* and isospores were determined using McMaster's method in Raynaud's modification, cryptosporidia by staining smears ,blastocysts by the method of ethyl acetate-formalin concentration, and direct microscopy. The recorded protozoa were species *Eimeria* spp .,*Cystoisospora suis*) syn .*Isospora suis* ,(*Balantidium coli* ,*Cryptosporidium* spp .,*Blastocystis* spp .These species of protozoa were observed in 31.1%, 49.0%, and 58.8% of pigs in large ,medium-sized, and small farms, respectively. The findings indicated that *Isospora suis* and *Eimeria* spp .were most commonly present in piglets aged 0-2 months in large (29.7% and 23.0% ,respectively), medium (32.3%, 29.4%), and small farms (30.0%, 13.5% .(*Balantidium coli* was not registered in pigs from large farms, and in piglets 2-4 months old from small farms, the prevalence of infestation was 17.2% .*Cryptosporidium* spp .and *Blastocystis* spp .were mostly recorded in piglets 2-4 months old from small farms (16.2% .(%7.1 ,In large and medium-sized farms of the Northern Black Sea region, mono infestations were recorded the most (73.6%, 72.0%), while mixed two-component infestation dominated (52.5%) small farms .Intestinal protozoa should be considered in the differential diagnosis of intestinal disorders as major factors or concomitant intestinal pathogens.
Keywords: Age, Animals, Black Sea, Intestinal protozoa, Prevalence

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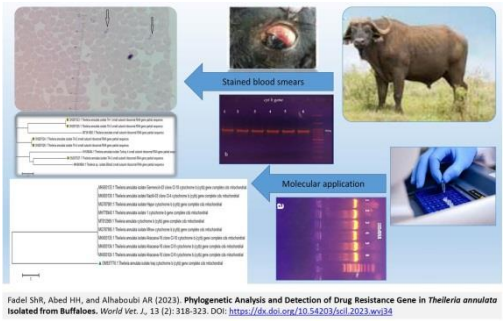
Research Paper

Phylogenetic Analysis and Detection of Drug Resistance Gene in Theileria annulata Isolated from Buffaloes

Fadel ShR, Abed HH, and Alhaboubi AR.

World Vet. J. 13(2): 318-323, 2023; pii:S232245682300034-13
DOI: https://dx.doi.org/10.54203/scil.2023.wvj34



ABSTRACT: Bovine theileriosis, caused by *Theileria annulate,* is disease affecting cattle and buffaloes worldwide. The current study aimed to screen the blood samples of 30 naturally suspected local buffaloes infected with *Theileria* species. The blood samples were initially examined by light microscopic and then the positive samples were subjected to PCR reactions. All 30 animals indicated clinical symptoms, such as high fever, loss of appetite, the presence of the hard tick, and enlargement of lymph nodes. The amplified products of *18S rRNA* were analyzed, along with molecular detection of the drug-binding site alterations and interrelated changes in the *cytochrome b* (*cyto b*) gene. Blood smears revealed the presence of infected erythrocytes with *Theileria* spp. The PCR results confirmed infection in samples when DNA amplified with partial *18S rRNA* and *cyto b* genes. The sequencing data were obtained from GeneBank using the accession numbers OM937770.1, ON207523.1, ON207525.1, ON207524.1, ON207526.1, and ON207527.1 Following BLAST analysis (Basic Local Alignment Search Tool), genetic differences were observed between the Iraqi isolate OM937770.1 and strains from India, Iran, and Turkey. The data obtained from the current study may reveal the genetic alteration of the local strain in the drug-target codons, which are found in one isolate and are different from the GenBank isolates. The results suggest that the failure of buparvaquone therapy might be due to the resistance to *cyto b* gene.
Keywords: Buffalo, Buparvaquone, Gene, *Theileria annulata*

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Research Paper

Protective Role of Rosa damascena Miller hydroalcoholic extract on Oxidative Stress Parameters and Testis Tissue in Rats Treated with Sodium Arsenite

AKhorasgani EM, and Mahdian Sh.

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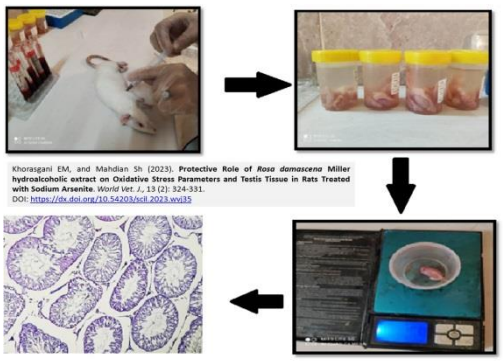

ABSTRACT: Regarding the strong antioxidant properties of Rosa damascene extract, this study aimed to investigate the protective role of Rosa damascene Miller hydroalcoholic petal extract on oxidative stress parameters and testis tissue in rats treated with sodium arsenite. To this end, 30 male rats were divided into five groups, including control, positive control (treated with arsenite), and three groups of patients affected by sodium arsenite with 150 mg/kg, 300 mg/kg, and 450 mg/kg Rosa damascene extract for 34 days by gavage. The animals were then anesthetized, and the blood samples were collected from the heart. The left testis was removed for histopathological studies. The findings revealed that Sodium arsenite in the positive group caused a significant reduction in TAC, testosterone, and serum Luteinizing hormone (LH) and a significant increase in serum Malondialdehyde. In addition, there was no statistically significant difference among the groups regarding the amount of Follicle-stimulating hormone (FSH). Moreover, the consumption of Rosa damascene extract with sodium arsenite caused a significant increase in testosterone, LH, and FSH compared to the positive control group. Histopathological results showed that in the experimental group receiving a dosage of 300 mg/kg b.w and the control group, the number of sperm tubes increased, and the germinal epithelium's thickness was appropriate. Daily treatment with Rosa damascene extract with a dosage of 300 mg/kg b.w for 34 days could improve the changes caused by sodium arsenite and reduce Malondialdehyde levels. Thus, it seems that Rosa damascene hydroalcoholic extract can effectively improve the male reproductive system's function.
Keywords: Oxidative stress, Rats, Rose petals, Sodium arsenite, Testis

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Research Paper

Protection of Khaki Campbell Ducks against Duck Plague Using an Inactivated Duck Plague Vaccine

Ahamed T, Sultana P, Rahman MZ, Bose P, Islam MR, Khatun MM, and Islam MA.

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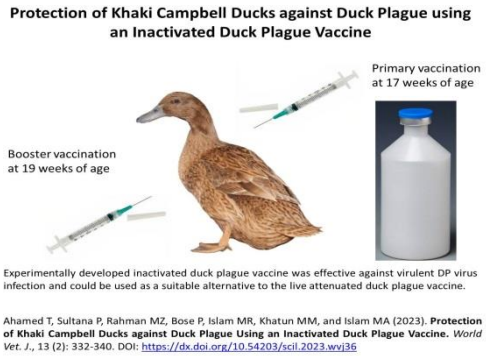

ABSTRACT: Duck plague (DP) or duck viral enteritis is a fatal viral disease of ducks that causes huge economic losses in the duck industry. The present study was performed to determine the immune response and protective efficacy of an inactivated DP vaccine prepared from a local virulent DP virus. A virulent DP virus was obtained from the laboratory repository of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh (Bangladesh). The DP virus ($EID_{50}/10^{5.3}$ml (was inactivated using 0.04% formalin. The alum (40 g/L) was added to the inactivated DP virus as an adjuvant. A total of 60 Khaki Campbell male ducks aged 17 weeks were randomly divided into three groups. Ducks of groups A (n = 20) and B (n = 20) were vaccinated intramuscularly in the breast muscle with 1 ml of inactivated DP vaccine and a live attenuated DP vaccine, respectively. Ducks of group C (n = 20) were kept as unvaccinated control. Booster vaccination was administered at 2 weeks after primary vaccination. Antibody titers of vaccinated ducks were measured at 7, 14, 21, and 28 days post-vaccination (DPV) using a passive haemagglutination (PHA) test. Ducks of both vaccinated and unvaccinated groups were challenged with 1 ml virulent DP virus ($EID/10^{4.3}$ $_{50}$ml (at 28 DPV. Clinical signs, morbidity and mortality, and gross pathological lesions of vaccinated and control ducks were observed for 10 days post-challenge to evaluate the protective efficacy of inactivated DP vaccine. The mean PHA antibody titers of vaccinated ducks of group A at 7, 14, 21, and 28 DPV were 5 ± 0.43, 26 ± 1.71, 43 ± 3.4 ,and 54 ± 3.28, respectively. Ducks in group B had mean serum PHA antibody titers of 21 ± 1.71, 41 ± 3.28, 52 ± 3.41, and 84 ± 7.25 at 7 ,21 ,14 ,and 28 DPV, respectively. No mortality or gross pathological lesions were observed in vaccinated ducks after they were subjected to a challenge infection. Additionally, no significant difference was observed between groups A and B in terms of the challenge infection. The mortality rate of the control group of ducks was 70%. Hemorrhage in the trachea and intestine and necrotic foci in the liver were seen in unvaccinated control ducks (group C). Experimentally developed inactivated DP vaccine induced a protective serum antibody titer and conferred 100% protection against virulent challenge infection up to 10 days observation period.
Keywords: Duck plague, Khaki Campbell, Protective efficacy

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Research Paper

Effects of Commercial and Homemade Extenders on Post-thaw Sperm Quality and Fertility of Semen from Ethiopian Indigenous Horro Chicken Breed

Getachew T, Goshu G, and Lemma A.

World Vet. J. 13(2): 341-347, 2023; pii:S232245682300037-13
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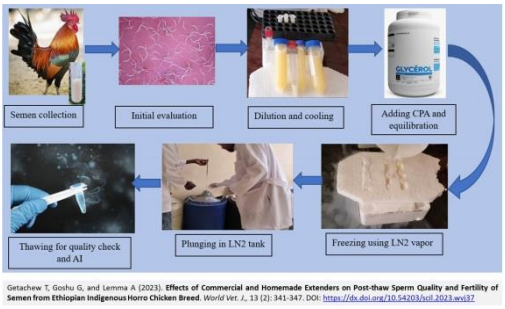

ABSTRACT: Cryopreservation of spermatozoa represents an important strategy for *in vitro* programs designed for the conservation of the genetic material of livestock populations. The objective of this study was to evaluate the effects of homemade tris-egg yolk-based and commercial poultry semen extenders on post-thaw sperm quality, fertility, and hatchability of semen from the Ethiopian Indigenous Horro chicken breed. A total of 30 roosters were used for semen collection, and 160 adult hens were inseminated artificially. The collected, qualified, and pooled semen samples were divided into three groups. Each semen sample was diluted with a homemade tris-egg yolk-based extender, Dimethyl-formamide commercial extender, and Commercial Beltsville Poultry Extender. Each extended semen was further divided into 20 sterile tubes as replicates. The extended semen samples were cryopreserved in liquid nitrogen using standard procedures. Changes in post-thaw spermatozoa mass and progressive motility, *in vitro* viability, morphological abnormality, fertility, and hatchability were evaluated. The post-thaw evaluation showed a decrease in the mass and progressive motility, morphologically normal spermatozoa, and an increase in dead spermatozoa and spermatozoa with bent necks, compared to fresh semen. There were significant differences in progressive sperm motility, motility, and *in vitro* viability between commercial and homemade cryoprotectants. However, no significant difference was observed in mass motility across the extenders. The commercial Dimethyl-formamide

extender was found to be the most suitable regarding the proportion of morphologically normal sperm and *in vitro* viability rate of cryopreserved sperm samples. There were no significant differences across all treatments in terms of fertility and hatchability rate. However, there was a significant difference between the control treatment and commercial extenders in terms of fertility and hatchability. The findings indicated favorable outcomes for a tris-egg yolk-based extender that was prepared locally with regard to the cryopreservation of poultry semen. Additional investigations are recommended to enhance the fertility and hatchability of the semen.
Keywords: Cryopreservation, Horro, *In vitro* viability, Morphology, Motility, Semen, Sperm

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Research Paper

Pathologic-anatomical Changes in the Comorbidity of Eimeriosis and Tuberculosis in Domestic Chicken and Decorative Pheasants (*Phasianus colchicus* L., 1758)

Liulin P, Bogach M, Lyakhovich L, and Ulyanizka A.

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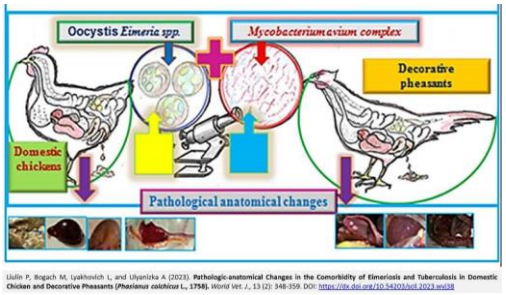

ABSTRACT: The study of patho-anatomical changes is essential in identifying pathological processes and diagnosing especially comorbid pathologies. The aim of this study was to reveal pathological changes and differences in the spontaneous comorbidity of tuberculosis (*Mycobacterium avium*) and eimeriosis (*Eimeria* spp.) in adult chickens and ornamental pheasants. The paper highlighted the results of pathological-anatomical changes in adult domestic chickens (n = 17) and ornamental pheasants (n = 5) with spontaneous comorbidity of eimeriosis and tuberculosis. Mycobacteria was detected using bacterioscopy of smears-prints from fragments of organs and *Eimeria* oocysts were detected by the Füllenborn flotation method. In pheasants, tubercular granulomas were found in the intestine, liver, and spleen in combination with scarring and swelling of the wall and mesentery, and venous stasis in the mesenteric vessels. In addition, hematomas and organ destruction in the liver and spleen were found in pheasants. In domestic chickens, tuberculous granulomas and steatosis were found in the liver. In the intestines, there were indications of mucocatarrhal inflammation, edema, hyperplasia, and small hemorrhages in the area of the cecal-intestinal diverticulum. Tuberculous nodules, internal hemorrhages around the perimeter of the tubercle, and devascularization were observed in the spleen. The present study revealed notable differences in the pathological and anatomical changes resulting from the comorbidity of eimeriosis and tuberculosis in domestic chickens and pheasants.
Keywords: Avian tuberculosis, Comorbidity, Eimeriosis, Intestine, Liver, Pathological change, Spleen

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Research Paper

Effects of Hydroxychloroquine and Tacrolimus on Discoid Facial Lupus Erythematosus in a Dog

Zhelavskyi M, Kernychnyi S, and Betlinska T.

World Vet. J. 13(2): 360-364, 2023; pii:S232245682300039-13
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ABSTRACT: Discoid lupus erythematosus is lupus in dogs an autoimmune disease that affects the skin. A 6-year-old, 38 kg, male German Shepherd dog was taken to the Small Animal Hospital at the University in Kyiv, Ukraine with a history of progressive skin lesions. The indications of discoid lupus erythematosus in dogs manifested as red, scaly macules or papules on the skin's surface. These gradually develop into follicular plugging, disc-shaped plaques with adherent scales, and peripheral hyperpigmentation. The oral hydroxychloroquine was used for medication and the prescribed dosage was 5.0 mg per kilogram of the dog's weight, administered once daily. The hydroxychloroquine was gradually reduced and discontinued within a month. Concomitant with the hydroxychloroquine treatment, the application of tacrolimus cream (Protopic® 0.03%) was initiated. The veterinary physician also advised the dog owner to limit sun exposure to avoid any adverse effects. After a four-week period, there was a decrease in pruritus and erythema, and plaques had flattened although the skin still had some patchy hyperpigmentation. Approximately 3-4 weeks later, the veterinarian determined

that the dog had achieved clinical remission as all the skin lesions had become completely flattened. The use of deproteinized calf blood extract gel (Solcoseril® Gel for external 4.15 mg/1 g, Legacy led to the complete disappearance of the initial redness and prevented the appearance of new skin lesions. These results can be considered as a safe and effective alternative to conventional treatment methods.
Keywords: Dog, Discoid lupus erythematosus, Treatment

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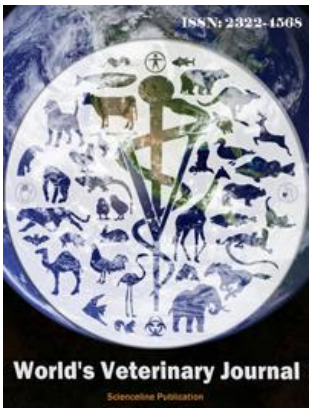

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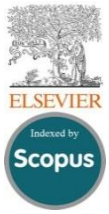

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Newcastle Disease Virus in Poultry: Current Status and Control Prospects

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REVIEW ARTICLE
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ABSTRACT

Since its first appearance in Java Island, Indonesia, in 1926, Newcastle disease has significantly impacted the global poultry industry, leading to substantial economic losses. The disease has rapidly spread worldwide, becoming endemic in many countries where agriculture is the primary source of national income. The present study aimed to present a comprehensive review of the recent literature on the Newcastle disease virus to contribute to understanding the virus and its control measures in poultry and provide an updated perspective on current knowledge. All strains of the Newcastle disease virus are classified under a single serotype; however, they are grouped into two classes and have been found to have emerging genetic diversity. Although various molecular diagnostic protocols have been developed, many have limitations. Nucleotide variability in the *F* gene of the Newcastle disease virus seems to explain the false-negative results provided by different real-time reverse transcription polymerase chain reaction protocols. Vaccination combined with biosecurity measures has been shown to limit the devastating effect of the Newcastle disease virus. However, the current vaccines are not effective enough to prevent viral shedding and infection of vaccinated animals. The efficacy of the vaccine strains utilized for decades is being scrutinized, raising questions about their effectiveness over time. The development of reverse genetics offers promising prospects for exploring new generations of attenuated vaccines capable of protecting poultry against clinical diseases and infections, such as Newcastle disease.

Keywords: Diagnosis, Genotype, Newcastle disease, Pathogenicity, Poultry, Vaccination

INTRODUCTION

Newcastle disease, or fowl plague, is a highly contagious viral poultry disease affecting various avian species with varying degrees of susceptibility (Shakal et al., 2020). The virus is easily transmitted, leading to its rapid spread worldwide within three decades after its initial appearance in Indonesia in 1926. Newcastle disease is currently endemic in many countries worldwide (Azizah et al., 2021). Based on pathogenic studies, the virus strains can be classified into five pathotypes, namely asymptomatic enteric, lentogenic, mesogenic, viscerotropic, and neurotropic velogenic strains (Abdisa and Tagesu, 2017; Getabalew et al., 2019). Velogenic strains are capable of causing 100% mortality in unprotected poultry farms, leading to trade restrictions and embargoes in countries where the disease occurs (Dzogbema et al., 2021; Ali et al., 2022). The disease can harmfully affect developing countries where agriculture is the main source of household income and food (Fellahi and Boudouma, 2021). Newcastle disease is a notifiable disease by the World Organization for Animal Health (WOAH). It is defined as any infection caused by an isolate of *Avian Paramyxovirus type 1* with an intracerebral pathogenicity index (ICPI) in day-old chicks of 0.7 or greater and having several basic amino acids at the C-terminal part of the F2 protein and phenylalanine at residue 117 of the N-terminal part of the F1 protein (WOAH, 2022).

Despite the use of vaccines to limit the devastating effects of the virus, Newcastle disease continues to affect the poultry industry worldwide, causing epidemics and significant economic losses (Hu et al., 2022). Although current vaccines can prevent the outbreak of the disease and death from the disease when administered properly, the virus can infect vaccinated animals and replicate, leading to its spread via feces and saliva to other birds (Miller and Koch, 2013; Bello et al., 2018; Mahamud et al., 2022). Recent years have seen a renewed interest in research on the virus due to its pathogenic potential and its use as a vaccine vector in both human and animal health (Jamil et al., 2022; Naz et al., 2022). Advances in molecular biology and genomic sequencing have greatly expanded the understanding of the biology and replication of the Newcastle disease virus (NDV). Furthermore, the development of reverse genetics offers prospects for the construction of a new generation of an attenuated vaccine capable of controlling both the clinical disease and its infection with regard to the modular nature of transcription, low frequency of recombination, and the absence of DNA phase during replication (Fellahi and Boudouma, 2021).

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The objective of this review was to provide a synthesis of recently published studies in the field. This review included the original studies conducted on the NDV, its virulence and pathogenicity mechanisms, genetic diversity, clinical and lesional picture resulting from its infection, and new methods and tools for laboratory diagnosis. The investigated articles were limited to those searched in the Google Scholar search engine. This review also examined the prevention and control methods currently in use and prospect.

Taxonomy and structure of Newcastle disease virus

Newcastle disease is caused by virulent strains of *Avian orthoavulavirus 1* (AOAV-1), formerly known as *Avian avulavirus 1* (AAvV-1) and commonly known as *Avian paramyxovirus 1* (APMV-1) or NDV. Newcastle disease virus belongs to the genus *Orthoavulavirus*, subfamily *Avulavirinae*, family *Paramyxoviridae* and order *Mononegavirales*. Isolated avian paramyxoviruses are classified into 21 serotypes designated APMV-1 to APMV-21. Each virus belongs to a viral species that are dispersed among the genera *metaavulaulavirus*, *orthoavulavirus*, and *paraavulavirus* (Amarasinghe et al., 2019; Dimitrov et al., 2019).

Newcastle disease virus is a pleomorphic enveloped virus sized approximately 200-300 nm in diameter. Its envelope is derived from the plasma membrane of the infected cell, with an outer face, where 8-12 nm long spicules are inserted, corresponding to the HN glycoprotein and the F protein. The inner side of the envelope is lined with a matrix protein. The viral genome is a non-segmented, single-stranded RNA of negative polarity. The RNA genome of NDV has a molecular weight of 5.2 to 5.7 X 106 Daltons. Genome sizes vary between 15186 and 15198 nucleotides (Ali et al., 2022). The nucleoprotein combines with the RNA to form a tubular nucleocapsid with a diameter of 18 nm, and the nucleoprotein, phosphoprotein, and large protein are tightly bound to the genomic RNA (Mao et al., 2022).

Viral proteins

The NDV genome consists of six genes, encoding six different proteins. The genes arranged in order of 3'-NP-P-M-F-HN-L-5' encode for nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and large polymerase protein, respectively (Phale, 2018; Nurzijah et al., 2022). The nucleoprotein has a length of 489 amino acids and a molecular weight of 55 kDaltons. It surrounds the genomic RNA and protects it from nucleases, while phosphoprotein is made up of 395 amino acids and forms multiple bands with a molecular weight ranging from 50 to 55 kDaltons, which is essential in viral replication and transcription. Matrix protein (M) is a non-glycosylated protein associated with the inner surface of the envelope. It consists of 364 amino acids and has a molecular weight of about 40 kDaltons. It plays an important role in viral assembly and envelope stabilization. Glycoprotein (F) consists of 553 amino acids and fuses the viral envelope with the membrane of the target cell. Glycoprotein (HN) is a 577 amino acid long polypeptide with a molecular weight of 74 kDaltons. It is a protein with many functions related to the attachment of the virion to the target cell receptor, enzymatic cleavage of sialic acid, and promotion of viral fusion. The large protein is the largest in the viral genome consisting of 2204 amino acids and has a molecular weight of 250 kDaltons. This protein synthesizes viral mRNA and assists in the replication of genomic RNA (Ganar et al., 2014; Phale, 2018; Fellahi and Boudouma, 2021). Accessory proteins V and W are proteins derived from the *P* gene translated from alternative mRNAs produced by RNA editing during *P* gene transcription. The V protein consists of 239 amino acids with a molecular weight of 36 kDaltons. It is an interferon antagonist and plays an important role in NDV virulence. Finally, the role of the W protein in the NDV replication cycle has not yet been established (Ganar et al., 2014; Nurzijah et al., 2022).

Viral replication

The spread of NDV in the infected organism occurs in several steps. It begins with the penetration of the virus into the host cell via surface glycoproteins (HN and F). The HN protein binds to sialic acid-containing cell receptors on the host cell surface, while the F protein mediates viral entry into the host cell. After the fusion of the viral envelope with the host cell membrane, the viral nucleocapsid is released into the cytoplasm, where the transcription and translation phase begin. During this phase, the negative-sense RNA genome is transcribed into positive-sense mRNA, which is then translated into viral proteins. The mRNA of individual genes is synthesized and used as a template for the synthesis of negative-sense genomic RNA. Transcription of genomic RNA into messenger RNA is carried out by the viral transcriptase (P and L) associated with the nucleocapsid. After the transcription and translation phase, the newly formed viral particles undergo budding and release. The M protein of NDV is essential for the assembly and budding of viral particles (Mao et al., 2022). Its nuclear localization could promote viral replication, increase viral RNA synthesis, and inhibit host cell replication (Duan et al., 2019). The F protein synthesized as a non-functional precursor F0 is cleaved into F1 and F2 by host cell proteases. Viral proteins synthesized in the host cell are transported and incorporated at the cell membrane. Once the nucleocapsids are incorporated into the modified regions of the cell membrane, new viral particles bud at the cell surface, taking with them part of the cytoplasmic membrane. Finally, the HN protein catalyzes the cleavage of sialic acid in the cell receptor and allows the release of the virus (Ganar et al., 2014; Mao et al., 2022).

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Infection can also occur by receptor-mediated endocytosis (Sánchez-Felipe et al., 2014). Cellular cholesterol may be required for optimal cell entry into the NDV infection cycle (Martín et al., 2012).

Resistance, virulence, and pathogenicity

The NDV can survive for long periods at room temperature (18 to 23.5°C), especially in feces (Getabalew et al., 2019; Dzogbema et al., 2021). However, it is inactivated at an elevated temperature of 56°C for 3 hours or 60°C for half an hour. It is also inactivated by an acidic pH ≤ 2. Finally, it is sensitive to detergents, lipid solvents, formaldehyde, and oxidizing agents (Getabalew et al., 2019).

The pathogenicity of NDV differs depending on the virus strain. It is well known that activation of the fusion protein (F) by host cell proteases is the primary determinant of virulence (Ganar et al., 2014). An inactive precursor glycoprotein (F0) is produced during replication. It must be cleaved into F1 and F2 polypeptides to exhibit virulence and become infectious (Worku and Teshome, 2020). The cleavability of the F0 molecule is directly related to the virulence of the virus. However, it can be influenced by the nature of proteases present in host tissues and organs. Molecular studies of the F0 glycoprotein site have revealed that the sequence of the F cleavage site between positions 112 and 116 primarily determines the virulence of NDV isolates (Puro and Sen, 2022). Virulent viruses are cleaved by furin-like proteases found in many tissues and organs, allowing them to cause lethal systemic infections. In contrast, trypsin-like proteases can only cleave low-virulence viruses in limited areas, such as the respiratory and intestinal tracts (Phale, 2018). According to Heiden et al. (2014), regions of the F protein modulate virulence other than the polybasic cleavage site.

The development of reverse genetics over the past two decades has allowed researchers to modify NDV genetically and study the individual contribution of genes and genome regions concerning its virulence (Dortmans et al., 2011). According to Samal et al. (2011), the conserved glutamine residue at position 114 in the cleavage site of the F protein is important in NDV replication and pathogenicity. In addition, the replacement of isoleucine at position 118 with valine around the fusion cleavage site reduces the pathogenicity of NDV. Additionally, mutation of the F protein glycosylation site can lead to a hyper fusogenic virus that could increase the virulence of NDV strains (Samal et al., 2012). Mutation of the cytoplasmic domain of the F protein can lead to the creation of a new hyperfusogenic virus that may increase viral replication and pathogenicity (Samal et al., 2013). Other studies have found that the amino acid residue located on site 430 of the HN protein of NDV can influence viral fusion capacity by promoting F protein cleavage (Chen et al., 2021). In addition, ubiquitination on lysine 247 of the NDV matrix protein enhances viral replication and virulence by promoting nuclear-cytoplasmic trafficking (Peng et al., 2022).

Genetic diversity and geographic distribution

Several classification schemes have been developed to identify and differentiate strains of NDV. Initially, these schemes were based on the biological properties of the virus (Diel et al., 2012). However, a new classification system has been proposed by Dimitrov et al. (2019), which is based on phylogenetic topology, genetic distances, branch support, and epidemiological independence of the virus. This system maintains two classes (I and II) of NDV, identifies three new class II genotypes, and reduces the number of subgenotypes. The class I NDV isolates are all grouped into a single genotype and three subgenotypes, as they exhibit strong genetic relatedness. These isolates have been identified in wild and domestic birds from Africa, Asia, Europe, and America (Rauw et al., 2009). It is worth noting that NDV isolates belonging to class I are generally considered to be of low virulence to chicken, with a few exceptions, such as an isolate that caused a severe epidemic in Northern Ireland in the early 1990s (Bello et al., 2018), as well as the JS10-A10 and 9a5b strains generated by consecutive experimental passages through chicken (Rehman et al., 2018).

Class II NDV isolates include viruses of varying susceptibilities, including lentogenic, mesogenic, and velogenic strains, resulting in nearly twenty genotypes due to ongoing virus emergence and evolution (Bello et al., 2018). The most prevalent genotype among all class II viruses in waterfowl is genotype I, which is apathogenic like nearly all class I viruses. Genotype I is further divided into three subgenotypes, namely 1a, 1b, and 1c (Rauw et al., 2009). Genotype II is neurotropic but includes viruses of different pathogenicity (lentogenic, mesogenic, and velogenic). Some viruses belonging to this genotype, including B1, LaSota, VG/GA, and strains not commonly isolated, are used as vaccines (Miller et al., 2010). Genotype III has been isolated mainly in Southeast Asia, Australia, Japan, the United Kingdom, Zimbabwe, Singapore, and China. All NDV isolates belonging to genotype III are virulent, and their isolates have been found in chicken and domestic waterfowl (Dimitrov et al., 2016). Genotype IV is associated with Newcastle disease epizootics in Europe after World War II (Wehmann et al., 2003). Genotype V includes four subgenotypes (Va-Vd) and was responsible for an epizootic wave that started in Western Europe and spread to Yugoslavia during the 1970s (Wehmann et al., 2003). This genotype has also been isolated in Central and North America and Africa (Denis et al., 2015). Genotype VI has 11 subgenotypes (VIa-VIk) and has been described in pigeons in Asia, Africa, Europe, and South America (Dimitrov et al., 2016; Bello et al., 2018). Genotype VII is commonly found in Asia and Africa (Xue et al., 2017; Naguib et al., 2021) and has been associated with the most recent Newcastle disease epizootics

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worldwide (Miller et al., 2015). This genotype has complex genetic diversity since it can be subdivided into 9 subgenotypes (VIIa-VIIi; Bello et al., 2018). Genotype VIII has been isolated from South Africa, South Asia, and western China (Cao et al., 2013; Denis et al., 2015; Megahed et al., 2020). Virulent and low-virulent isolates of genotype IX have been described in several asymptomatic wild bird species in China (Duan et al., 2014). Virulent strains of genotype X have been identified in Taiwan, Argentina, and the USA. Genotype XI is also virulent, but its distribution is largely restricted to the island of Madagascar (Maminiaina et al., 2010). Genotype XII has been isolated in South America and China (Liu et al., 2013; Chumbe et al., 2017), while XIII genotype includes three subgenotypes (XIIIa-XIIIc) and has been identified in Asia, Europe, and Africa (Denis et al., 2015; Das and Kumar, 2016; Ana et al., 2020). Genotype XIV includes two subgenotypes and is highly virulent, and has only been found in domestic birds in Africa (Snoeck et al., 2013; Samuel et al., 2013). Genotype XV was isolated in China and included virulent and vaccine strains (Bello et al., 2018). Genotype XVI is strongly related to genotype IV and has been isolated in Europe, Africa, and Asia (Bello et al., 2018). Finally, genotypes XVII and XVIII have two subgenotypes, and they are highly virulent and are found in West and Central Africa (Snoeck et al., 2013; Bello et al., 2018; Souley et al., 2021).

Host species

Newcastle disease virus is capable of infecting over 200 different bird species, but the disease infection consequence varies greatly depending on the host species and the virus strain (Rauw et al., 2009). Newcastle disease affects chickens more severely than turkeys, which typically show few clinical signs (WOAH, 2022). Pheasants, partridges, quail, and guinea fowl are also susceptible (Getabalew et al., 2019). Pigeons (Colombiformes) can also be infected with pigeon paramyxovirus (PPMV-1, Ramsubeik et al., 2023). Ducks and geese (Anseriformes) may be infected but show little to no clinical signs (Rahman et al. 2018). Teal, swans, wild geese, double-crested cormorants, white pelicans, and gulls are susceptible, but they are generally resistant and show little clinical signs of apathy and anorexia (Rasamoelina et al., 2016). Parrots, ravens, sparrows, and kingbirds are susceptible (Rahman et al., 2018). Generally, wild birds and waterfowl are considered reservoir hosts (Getabalew et al. 2019). Newcastle disease can also infect a range of non-avian species. Evidence of Newcastle disease infection and detection have been reported in cattle, sheep, mink, hamsters, mice, rabbits, camels, pigs, monkeys, and humans (Ul-Rahman and Shabbir, 2019; Shabbir et al., 2021; Ul-Rahman et al., 2022). Workers in poultry production and processing and vaccine production laboratories are at the greatest risk (Shabbir et al., 2021).

Mode of dissemination

Transmission of NDV can occur through inhalation or ingestion of virus particles. In the case of natural infection, the virus multiplies in the respiratory and/or digestive tract of infected poultry, which then excretes the virus through airborne or fecal routes (Brown and Bevins, 2017). These released virus particles are inhaled by healthy birds or affect their mucous membranes. Viruses released in feces may also contaminate feed and drinking water and thus be ingested by other birds in the poultry house (Rauw et al., 2009). Non-bird species, such as cats, dogs, foxes, and rodents, may play a role in virus transmission because they may spread the virus for up to 72 hours in their feces after ingesting contaminated poultry (Rasamoelina et al., 2016). Similar to insects, rodents, and reptiles may also be potential vectors of NDV, as their susceptibility to infection has been reported (Rasamoelina et al., 2016). Semi-captive or free-living wild birds may act as natural reservoir hosts for NDV and play a considerable role in the spread of the virus (Rahman et al., 2018).

Clinical signs and lesions

Pathogenicity of the virus strain, host species, host age, secondary infections, stress, environmental conditions, host immune status, viral dose, and route of exposure may play a role in determining incubation time and disease severity (Getabalew et al., 2019; WOAH, 2022). Infection with lentogenic NDV virus strains can range from unapparent respiratory or gastrointestinal illness to mild disease in adult chickens. Infection with mesogenic isolates can lead to the development of nonfatal respiratory disease, decreased egg production, and rare nervous signs, but mortality is generally low (Abdisa and Tagesu, 2017). Infection with highly virulent velogenic viruses can cause mortality in highly susceptible flocks of up to 100% (WOAH, 2022). Viscerotropic velogenic strains cause acute infection of the gastrointestinal mucosa and result in hemorrhagic lesions and death. Clinical signs resulting from chicken infection include general ill health, respiratory disturbances, greenish or whitish diarrhea, muscle tremors, and paralysis of the extremities. Edema may also be seen around the eyes (Caroline, 2022). Neurotropic velogenic strains of NDV primarily cause respiratory distress followed by neurological disease and decreased egg production (Abdisa and Tagesu, 2017). Mortality in chickens infected with velogenic NDVs can reach 100% (Alazawy and Al Ajeeli, 2020).

Newcastle disease virus lesions are primarily characterized by hemorrhages and ulcerations in the proventriculus and intestine (Rauw et al., 2009). Infection with viscerotropic velogenic NDV can result in severe clinical signs in chickens, including dehydration and emaciation of the chicken carcass, hemorrhagic lesions, and necrotic ulcerations at

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the tips of the proventricular glands, in the intestine, caecal tonsils, and spleen parenchyma. Additionally, the lungs may show congestive, edematous, and hemorrhagic lesions (Mariappan et al., 2018; Sonkusale et al., 2023). In the respiratory form of the disease in chicken, lesions are marked initially by congestive or hemorrhagic tracheitis that becomes complicated after a few days of consecutive colibacillosis evolution into fibrinous tracheitis, fibrinous pneumonia, aerosaculitis, pericarditis and perihepatitis (Fellahi and Boudouma. 2021). The involvement of the central nervous system in NDV infection can be observed through anatomical manifestations, such as progressive encephalitis, neuronal necrosis, neuronal phagocytosis, the presence of clusters of cells of microglial morphology in the gray matter of the brain, cerebellum and spinal cord, axonal degeneration, and demyelination lesions (Ecco et al., 2011).

Diagnose

Observed clinical signs cannot provide a reliable basis for diagnosis, but can be used as an element of suspicion to guide the diagnosis (Abdisa and Tagesu, 2017). Lesions may also be confused with those of other diseases, including Avian Influenza (Ganar et al., 2014). However, a definitive diagnosis of Newcastle disease is based on the direct detection of viral antigens, which can be achieved by virus isolation from swabs of live animals or organs taken from cadavers. Virus culture is readily performed on 9-11-day-old embryonated chicken eggs or a wide range of cells, including chicken embryo fibroblast cells, chicken embryo hepatocytes, African vervet monkey kidney cells, and chicken embryo reticulum cells (WOAH, 2022). After incubation at 37°C for 4-7 days, the inoculated embryonated chicken eggs are refrigerated at +4°C. The allantoic-amniotic fluid from the eggs is then tested for NDV using the hemagglutination test. Confirmation of NDV infection can be done by performing a hemagglutination inhibition test or by using molecular methods (WOAH, 2022). Agar gel immunodiffusion technique, fluorescent antibody test, hemolysis test, or identification of viral particle morphology by electron microscopy can also be used to identify NDV, but none of them provide information on the pathotype of ND (Cattoli et al., 2011).

Serological diagnosis provides indirect evidence of NDV through antibodies that demonstrate infection. The hemagglutination inhibition (HI) test is the most widely used reference and confirmatory test for Newcastle disease serology (WOAH, 2022). The test is based on the agglutination of chicken red blood cells caused by the hemagglutinin of the viral envelope and the inhibition of hemagglutination by specific antibodies. Enzyme-linked immunosorbent assays (ELISAs) are also commonly used for the detection and quantification of antibodies. The antibody detection technique consists of binding antibodies to viral antigens on a microtiter plate. The viral antigens are captured by other detection antibodies produced in another species against those of the chicken and coupled to an enzyme that catalyzes the reaction to cause a color change. The plate is then read on a spectrophotometer (Dzogbema et al., 2021). The HI test correlates well with the ELISA test (Getabalew et al., 2019; WOAH, 2022). An intracellular cytokine labeling technique was developed using Pinette et al. (2014) to quantify the immune response mediated by T cells. There are other serological tests, such as the neutralizing antibody test, the immunofluorescence test, and the colloidal gold immune technique. However, the limitation of serological tests lies in the cross-reactivity between NDV and other homologous avian paramyxoviruses, which can give false positive results (Mao et al., 2022).

Techniques based on molecular biology methods have been developed for the isolation and identification of NDV strains (Dzogbema et al., 2021; Moa et al., 2022). Several laboratory protocols have been developed, including gel-based reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, restriction enzyme-based methods, and rapid sequencing. In addition, nested PCR, fluorogenic probe-based real-time RT-PCR, ligase chain reaction (LCR), intercalated DNA (SYBR Green), or light-extended fluorogenic primer (LUX) should also be mentioned. All of these assays have shown promising results, but most of them have some limitations and their ability to detect different virus types needs validation (Cattoli et al., 2011; Mao et al., 2022). Since the NDV *F* gene is the primary determinant of NDV pathogenicity, RT-PCR protocols to pathotype NDV generally target this gene (Putri et al., 2017; Abd Elfatah et al., 2021; Mao et al., 2022). The growth of the gene sequence database over the last decade has revealed the high degree of nucleotide variability in the NDV *F* gene, which is mainly responsible for mismatches between oligonucleotides (primers and/or probes) and cDNA templates. This may well explain the false negative results provided by different real-time RT-PCR protocols (Cattoli et al., 2011). Another real-time reverse transcription isothermal loop amplification (RT-LAMP) method has recently been developed. This assay is faster and more sensitive than real-time RT-PCR but had specificity only for NDV genotype VII (Selim et al., 2022).

Pathogenicity testing

Based on calculations of the intracerebral pathogenicity index (ICPI) in day-old chicks, the intravenous pathogenicity index (IVPI) in six-week-old chickens, or the mean time to death in embryos. The NDV isolates can be characterized as virulent (velogenic), moderately virulent (mesogenic), or less virulent (lentogenic). However, ICPI appears to be the more accurate and sensitive method compared to the other two methods (Dortmans et al., 2011). The calculation of ICPI is done after intracerebral infection of day-old chicks with a score ranging from 0 to 2, where 0 is assigned to a normal chick, 1 to a sick chick, and 2 to a dead chick. This score is assigned daily to each chick for eight

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days. Finally, the virulence of the virus strain is determined by the average of the scores. Strains are velogenic when the ICPI value is greater than 1.5. They are mesogenic when the ICPI value is between 0.7 and 1.5. Finally, they are lentogenic when the ICPI value is less than 0.7. The IVPI is calculated in the same way as the ICPI, but the six-week-old birds are injected intravenously. The presence of a velogenic strain is indicated by an IVPI greater than 2.5 (Al-Shammari et al., 2020). Mean time to death refers to the average time in hours required for all inoculated embryos to die. If the embryos die within 60 hours, it is an indication of the presence of velogenic strains, while survival of the embryos beyond 90 hours indicates the presence of lentogenic strains (Cattoli et al., 2011). *In vitro*, NDV induces plaque formation in embryonic fibroblast culture, the size and morphology of which varies according to the virulence of the viral strain. The amino acid sequence of the F protein cleavage site can also be used in differentiating the virulence of NDV isolates (Awad et al., 2020).

Control

Control of Newcastle disease must include strict biosecurity that will prevent velogenic virus strains from coming into contact with poultry and adequate administration of effective vaccines (Dimitrov et al., 2017). In addition, serological monitoring of the immunological status of vaccinated flocks should be performed to assess the antibody response to the administered vaccines (Ahmed and Odisho, 2018). Traditional vaccines (live and inactivated vaccines) have been widely used for several decades (Dimitrov et al., 2017). However, another generation of vaccines, such as recombinant vaccines and antigen-matching vaccines, have recently been adopted in some countries, and other vaccine approaches are in the experimental phase (Dimitrov et al., 2017; Nurzijah et al., 2022). The vaccine strains used are obtained from the naturally apathogenic, low pathogenic, or medium pathogenic strains (Dimitrov et al., 2017; Hu et al., 2022). These vaccine strains belong to genotype I (Ulster and V4), genotype II (Lasota and B1), genotype III (Mukteshwar), genotype IV (Herts/33, UK), genotype V (Anhinga, USA), genotype VIII (AF22440), and genotype XV (vaccine strains, China, Bello et al., 2018).

With the development of genetic engineering, NDV vaccine strains have emerged as promising vectors for developing effective multipurpose vaccines against pathogens that infect both animals and humans (Kim and Samal, 2016; Choi, 2017). For example, the use of an NDV virus-like particle-based vaccine expressing NDV F protein and influenza virus matrix 1 (M1) protein could protect chickens from a lethal challenge, while significantly reducing viral shedding and can also be used as a DIVA strategy to differentiate infected from vaccinated animals (Park et al., 2014). An experiment conducted by Izquierdo-Lara et al. (2019) indicated that vaccine strains paired with wild type XII strains conferred better protection to vaccinated animals and significantly reduced viral shedding. Similarly, Miller et al. (2013) in an experimental setting showed that by increasing the homology of the vaccine to a wild-type virus, humoral antibody efficacy levels could be increased. As the level of humoral antibodies increases in vaccinated birds, the number of infected birds and the amount of virus shed decreases. Results of an experiment conducted by Pandarangga et al. (2022) also revealed that viral shedding in chickens is reduced when vaccinated with strains homologous to the infectious challenge strain compared to the vaccine using the heterologous strain (LaSota).

Furthermore, in an intranasal vaccination trial in chickens, Zhao et al. (2016) found that a DNA vaccine containing the NDV *F* gene encapsulated in hollow nanoparticles induced higher serum antibody (IgA) titers. Results of a comparative study by Zhao et al. (2012) also showed that a live vaccine (LaSota strain) encapsulated in chitosan nanoparticles (NDV-CS-NP) induced better protection to immunized specific pathogen-free chickens, compared to the live LaSota strain vaccine and the inactivated NDV vaccine. An encapsulated inactivated NDV vaccine in chitosan nanoparticles has also been successfully developed (Mohammadi et al., 2021). These studies lay the foundation for the future development of mucosal vaccines encapsulated in nanoparticles. Plant-derived vaccines are in development and offer potential advantages over traditional vaccines, including a significant reduction in viral shedding and the ability to differentiate infected birds from vaccinated birds (Nurzijah et al., 2022; Smith et al., 2023).

CONCLUSION

Newcastle disease is a health threat to the global poultry industry. It is endemic in many developing countries. In disease-free countries, NDV outbreaks are occasional, but when they occur, they result in significant trade restrictions and embargoes. Newcastle disease is sometimes misdiagnosed because serological and molecular diagnostic tests for NDV have certain limitations as a result of cross-reactivity of NDV with other homologous avian paramyxoviruses and variability in the nucleotide sequence of the NDV *F* gene. To prevent ND, only sanitary and medical prophylactic measures can be used effectively to enhance the immune capacities of birds and reduce ambient viral pressure. The international reports show that vaccinated poultry can be infected with wild viruses. The NDV genotypes are emerging, which can make control strategies complex and often difficult, hence the need to use high-quality and up-to-date vaccines. Research priorities should therefore focus on improving diagnostic tools and developing better vaccines.

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Authors' contributions

This work was carried out with the contribution of all authors. Ahamidou Moustapha drafted the manuscript. Essodina Talaki, Akourki Adamou, and Moumouni Ousseini revised the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

The authors of the current study checked for ethical issues, including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy.

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Data availability and materials

Data from the study are available according to a reasonable request.

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Management Updates on Prepartal Stress Effects on Transition Cow and Calf Health

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ABSTRACT

The transition phase is thought to be the most critical period in high-producing dairy cows' productive cycle. Maternal stresses during the peripartum period affect both dam and newborn calf health. The objective of this review article was to describe the role of prepartal metabolic and environmental stressors on postpartum-related disorders in both cows and newborn calves. The transition phase (21 d before to 21 d after calving) is considered the most critical period in dairy cattle life. Decreased dry matter intake coincides with increased nutrient demands due to the onset of lactation. It can lead to negative energy balance and tremendous metabolic challenges for high-producing dairy cows. During this time, insulin concentrations and peripheral tissue sensitivity decrease, leading to fat mobilization from adipose tissue. Such incidences would increase levels of non-esterified fatty acids and beta-hydroxybutyric acid in plasma. The success in the transition from the dry to lactating phase depends on how the cow could deal with such challenges. Failure to handle such metabolic alterations may predispose cattle to costly metabolic disorders such as ketosis, fatty liver, retained placenta, displacement abomasum, and infectious diseases, including metritis and mastitis in the postpartum period. The energy content of dairy cow diets in the dry-off (40 days before calving) or close-up (21 days before calving) periods may influence cow physiology and health in the peripartum period. The role of a transition period diet on cow health and productivity in the early or overall lactation period has been frequently investigated. However, the role of stressors such as nutritional deficiencies, heat stress, stocking density, and grouping in the late gestation period on the health and performance of cows and calves are much less addressed. Therefore, the present review delineates and reviews updates on the role of maternal stresses during the peripartum period on dam as well as newborn calf physiology and behavior.

Keywords: Immunity, Management, Metabolic disease, Prepartal stress, Transition cow

INTRODUCTION

The transition phase (21 days before to 21 days after calving) is the most critical period in the high-producing dairy cows' productive cycle (Huzzey et al., 2005). A successful transition from a dry to lactating period is associated with delivering a healthy calf with the minimum occurrence of metabolic (displacement abomasum, ketosis, fatty liver, and milk fever) and infectious diseases (such as mastitis and metritis) for the dam in the postpartum period. Reduced feed intake and impaired immune function, alongside increased nutrient demands due to the onset of lactation, are the major challenges dairy cows face during the transition period (Lopreiato et al., 2020). Numerous studies on the transition period have mostly focused on dry matter intake (DMI) and negative energy balance (NEB) as the main factors determining cow health and subsequent milk production (Hayirli et al., 2002; Grummer et al., 2004; Pérez-Báez et al., 2021). However, the role of the immune function and welfare of prepartum cow on both dam and neonatal calf has been much less addressed in the literature or practice on farms.

Understanding the biology of high-producing dairy cows in the periparturient period may provide practical insights into the optimal management of this critical phase. As calving approaches, dairy cows undergo tremendous metabolic alterations to support fetus growth and subsequent milk production (Allen and Piantoni, 2013). Insulin concentrations and sensitivity of adipose tissues and muscles to this anabolic hormone decrease in the prepartum period, resulting in lipolysis that can be extended to the postpartum period (Allen and Piantoni, 2013). On the other hand, it seems that increased growth hormone (Balogh et al., 2008) and pro-inflammatory cytokines levels play a significant role in inducing insulin resistance in both humans (de Luca and Olefsky, 2008) and bovine adipocytes (Du et al., 2022). Lipolytic status in cows during the periparturient period leads to elevated levels of non-esterified fatty acids (NEFA) in plasma. Non-esterified fatty acids released from adipose tissue are taken up by the liver, which can be partially oxidized to ketone bodies, such as beta-hydroxybutyrate (BHBA) or stored as triacylglycerol (Grummer, 2008). The elevated levels of

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NEFA and BHBA in the periparturient period are likely associated with postpartum disorders such as ketosis and fatty liver (Overton et al., 2017). Some scientists recently indicated that immune dysfunction and inflammation are key factors causing depressed feed intake and related metabolic and infectious diseases postpartum (Horst et al., 2021). In addition, it is believed that stressors, such as heat stress, can worsen the situation with negative impacts on immune function (Mezzetti et al., 2021). According to the new findings, a comfortable environment with minimal stress could be a preventive strategy against postpartum metabolic and infectious diseases on-farm (Mezzetti et al., 2021).

The importance of stress during the prepartum period has been emphasized recently (Chebel et al., 2016; Mezzetti et al., 2021). Metabolic stresses in the peripartum period reduce feed intake and NEB in the postpartum period. The NEB is expected to be exacerbated when cows are exposed to stressful conditions. Maximizing feed intake of high-producing dairy cows in the prepartum period was an outstanding theory regarding transition cow management for many years (Drackley, 1999; Grummer et al., 2004). However, academic research and practical observations indicated that postpartum complications are more serious than expected (Drackley, 1999; Grummer et al., 2004). As such, the role of other management factors on the welfare of the prepartum cow was obtained. With respect to the growing number of studies investigating peripartum cow immunity and related postpartum issues, the present review aimed to offer updates on some serious practical management factors (such as nutritional management, stocking density, and regrouping) influencing transition cow health and early lactation disorders.

Traditional and current perspectives on transition cow biology

The transition period has a vital influence on the lactation period in dairy cows after parturition. This period is characterized by tremendous physiological alterations in hormonal activity and nutrient partitioning to help nourish the growing fetus and support the onset of new lactation (Allen and Piantoni, 2013). Growth hormone and somatotropin levels are increased, whereas circulating insulin levels and sensitivity of tissues to this hormone are decreased, leading to the mobilization of fatty acids from adipose tissue (Drackley, 1999). Non-esterified fatty acids are then used as an energy source by the liver and other peripheral tissues sparing glucose for fetus growth and lactose synthesis in the mammary glands (Allen and Piantoni, 2013). Because feed intake is suppressed naturally in prepartum cows, mainly over the two weeks pre-calving, glucose produced by the liver is limited due to inadequate gluconeogenic precursors derived from daily feed intake (Horst et al., 2021). Despite the benefits of NEFA as a fuel for the peripheral tissues, higher levels of NEFA and BHBA can lead to fatty acid deposition in the liver (fatty liver syndrome) or ketosis postpartum (Horst et al., 2021). Traditionally, the elevated levels of NEFA and BHBA accompanied by hypocalcemia are believed to be the main factors associated with the incidence of metabolic and infectious diseases postpartum (McArt et al., 2013). However, recent studies have argued that the association between NEFA and BHBA concentrations and the incidence of postpartum metabolic disorders are inconsistent, implying that other factors could be responsible (Pascottini et al., 2020; Mezzetti et al., 2021; Horst et al., 2021). In this regard, the role of immune dysfunction and pro-inflammatory cytokines (tumor-necrosis factor α, interleukin 1 and 6) in the etiology of early lactation disorders has been widely emphasized.

Moreover, the traditional transition period (3 weeks before to 3 weeks after parturition) may be modified from the current new perspective, given that metabolic adaptations may originate from the dry-off period (Pascottini et al., 2020). In this case, NEFA, cortisol, and positive acute phase proteins (such as haptoglobin) levels seem to be high following two days of drying off (Putman et al., 2018). The systemic inflammation occurring in the dry-off period might be the most important contributor to developing immune dysfunction and inflammatory status in the transition period (Mezzetti et al., 2021). However, the immune system inadequacy is remarkably initiated in 2 or 3 weeks relative to calving as the maximum immune dysfunction occurs at the time of calving until two days post-calving (Vlasova and Saif, 2021). It is believed that reduced polymorphonuclear cells and lymphocytes function accompanied by systemic inflammation would predispose dairy cows to metabolic and infectious diseases in early lactation (Biswas and Lopez-conzalo, 2009; Mezzetti et al., 2021). In a recent study to evaluate the relationship between prepartum immune function and sub-clinical ketosis (SCK) in early lactation, the authors found inflammatory status, altered immune function, and impaired kidney and liver function in the affected animals (Mezzetti et al., 2019). Furthermore, interferon-γ (INF-γ) levels were greater in the SCK cows. The greater INF-γ levels were related to increased insulin resistance and elevated levels of blood glucose, NEFA, and BHBA prepartum. As such, the increased circulating glucose coincided with increased energy needs for immune activation may exacerbate NEB (Horst et al., 2021). The severity of reduced immune competence and inflammatory status around calving may determine the magnitude of metabolic and infectious diseases in early lactation (Mezzetti et al., 2021). In light of the increasing scientific evidence regarding the role of immune adequacy during the prepartum period on the etiology of postpartum disorders, mitigating stressors in the dry period (60 d before expected calving) may prevent further immune challenges in transition cows.

Physiology of stress and animal response

Despite the beneficial effect of stress in helping animals escape from threats, it can be a harmful physiological situation affecting animal welfare and productivity (Edris and Feki, 2021). Earlier, stress was defined as a condition in which the sympathy-adrenomedullary (SAM) system is activated to regulate body homeostasis in response to

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various stimuli (Cannon, 1925). This system responds quickly to the stimuli leading to epinephrine and norepinephrine secretions from the adrenal medulla (Godoy et al., 2018). Epinephrine and norepinephrine would result in elevated levels of glucose and free fatty acids in the blood because of the catalytic nature of such hormones (Godoy et al., 2018). Moreover, epinephrine and norepinephrine stimulate gluconeogenesis. In addition, the hypothalamic-pituitary-adrenal axis (HPA) is actively involved when animals are exposed to internal or external stressors (Godoy et al., 2018). Social contacts, stocking density, cold and hot environments, nutritional deficiencies, transportation, weaning, as well as calf dehorning are the common stressors for dairy cows provoking the HPA axis and subsequent physiological events potentially (Godoy et al., 2018). The SAM and HPA systems are interrelated and considered the main parts of the adaptive response in stressed animals. Activation of the HPA axis would increase blood glucocorticoids, mainly cortisol (Wadsworth et al., 2019). Cortisol is also known as the main stress hormone. Corticotrophin-releasing factor (CRF) is secreted from the paraventricular nucleus (PVN) of the hypothalamus and stimulates the anterior part of the pituitary gland. Consequently, adrenocorticotropic hormone (ACTH) is released into the blood stream. Then, the ACTH hormone targets the adrenal cortex to release glucocorticoids (Godoy et al., 2018). It has been reported that corticosteroids released during stressful conditions potentially regulate many metabolic actions, such as immune and behavioral responses (Burdick et al., 2011).

It is important to note that stress can be classified into two main acute or chronic forms (Salak-Johnson and McGlone, 2007). The adaptation is quick and complete in the acute form, allowing physiological reactions to be balanced (Grelet et al., 2022). However, in the chronic form, the stress is long-lasting and continuous, leading to disrupted physiological adaptations, environmental resistance, and exhaustion (Grelet et al., 2022). It is believed that prolonged exposure to chronic stress may lead to impaired metabolic, endocrine, and immune status, compared to acute stress (Salak-Johnson and McGlone, 2007; Trevisi and Bertoni, 2009). In dairy cows, a recent cohort study indicated that prolonged exposure to stress could influence welfare, health, and production (Grelet et al., 2022). In a 4-week stressful period (overstocking and regrouping of cows), greater milk loss and lower rumination were observed in the stressed cows. Moreover, elevated hair cortisol and blood fructosamine levels were proposed as biomarkers of chronic stress in dairy cows (Grelet et al., 2022). It has been reported that glucocorticoids and catecholamines secreted during stressful situations may suppress the immune function, predisposing the host to various diseases (Salak-Johnson and McGlone, 2007; Trevisi and Bertoni, 2009; Burdick et al., 2011). In human studies, chronic exposure to higher levels of cortisol is associated with immune system resistance and additional production of inflammatory cytokines compromising the immune response (Morey et al., 2015). In farm animals, the risk of bovine respiratory disease increases following weaning or transportation (Kumar et al., 2012). In addition, reproductive performance and growth can be negatively affected by stressors existing in animals' environments (Kumar et al., 2012).

Unfortunately, the negative effects of stress are not limited to the animal herself. In pregnant animals, especially those in late gestation, the growing fetus could be negatively affected by environmental stressors, which can be extended to the postnatal period (Merlot et al., 2013). Dairy cows suffer from metabolic stresses, lipid mobilization, immune activation, inflammation, and oxidative status during the periparturient period, causing postpartum metabolic and infectious diseases (Ling et al., 2018). Such alterations may have carryover effects on the health and welfare of neonatal calves (Ling et al., 2018). The immune function of a dam exposed to environmental stressors is impaired, influencing the neonate's health (Merlot et al., 2013). It is believed that the origin of the disorders that the offspring face later in life may be explored in the prenatal period (Fowden et al., 2006; Reynolds and Caton, 2012). Such a process is called fetal or intrauterine programming that occurs at gene, cell, organ, and system levels resulting in fundamental changes in the physiology of neonates, and may lead to metabolic and infectious diseases in the future (Fowden et al., 2006). For instance, diarrhea, the most important disease-causing mortality in newborn calves, is the most prevalent disease in calves born to underfed or stressed mothers (Nikkhah and Alimirzaei, 2022). It has recently been reviewed that stressors such as malnutrition and heat stress during late pregnancy may have prominent effects on both dam and neonate immune and organ development (Osorio, 2020). In this case, the intestinal development and the ability of the epithelial cells to absorb colostrum IgG may be disrupted (Osorio, 2020). In addition, calves born to cows that were cooled in the late gestation had greater birth and weaning weights and tended to have greater average daily gain (ADG) than the group during heat stress (Lay et al., 1997). Calves exposed to stress (repeated transportation) in the prenatal period exhibited greater heart rate and cortisol levels (Lay et al., 1997). Such calves were less able to respond to stress than the other groups, resulting in reduced welfare. Generally, it appears that the immunity and health status of both dam and offspring could be affected by maternal stress in the late-gestation period. Therefore, minimizing stress in the dry cow environment would be a perfect management decision to transition from dry to milking phase optimally.

Nutritional deficiencies, stocking density, high and low ambient temperatures, transportation, and regrouping are among the most important stressors affecting dry cow and calf health and welfare negatively. The effects of these factors on cow and calf physiology and health are discussed below.

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Late-gestation stress sources

Nutritional management

A successful transition from the dry period to lactating period in a dairy cow needs metabolic adaptations to support fetal development and milk synthesis with the onset of the new lactation cycle (Pascottini et al., 2020). Failure to adapt to such metabolic and hormonal changes, especially during the last 3 weeks of gestation, may lead to postpartum health disorders (Pascottini et al., 2020). In addition to the metabolic stresses that high-producing dairy cows face during the transition period, oxidative stress may be another challenge for transition cows with the potential to compromise their immune function (Sordillo and Aitken, 2009). Nutritional and management strategies of the periparturient cow are the most important factors influencing dairy cows' health and reproductive performance (Janovick et al., 2022). Energy intake during the prepartum period is critical for the liver function and susceptibility of dairy cows to metabolic diseases postpartum (Janovick et al., 2022). Recently, it was demonstrated that overnutrition in the dry period would compromise liver function and genes related to peroxisome proliferator-activated receptors (PPAR) in the early lactation phase (Janovick et al., 2022). A recent study recommends restricting energy intake to 1.3-1.39 Mcal of NEl/kg of DM (Cardoso et al., 2020). The advantages of energy-restricted feeding strategies seem related to lower blood NEFA, BHBA, and liver triglyceride levels in the postpartum period (Mann et al., 2015).

Accumulation of lipids in the liver may be associated with inflammation in the periparturient cow. It has been illustrated that mRNA for serum amyloid A, an acute phase protein, was upregulated (increased) during the periparturient period, which may be related to postpartum disorders (Loor et al., 2005; Horst et al., 2021). Moreover, lipid accumulation in the liver of late lactation cows was reported following TNF-α administration (Drackley and Cardoso, 2014). Overall, a review of articles investigating the transition of cow health would propose that nutritional and management strategies must be adjusted toward attenuating excessive lipid mobilization and inflammation. Besides controlling the energy intake of dry cows, the body condition score (BCS) should also be monitored to avoid cow's fattening. Over-conditioned animals may reduce feed intake and mobilize fatty acids more than those with lower BCS (Drackley and Cardoso, 2014). As such, these cows are believed to have impaired immune function and elevated inflammation biomarkers (Drackley and Cardoso, 2014). As a practical guideline, the optimal BCS is about 3 out of 5 (Drackley and Cardoso, 2014).

In addition, micronutrient deficiencies due to increased animal requirements and fetus growth in the late gestation period seem to be a risk factor. It is well known that transition cows may suffer from oxidative stress that can affect their immune function negatively and increase micronutrient requirements (Sordillo and Atiken, 2009). It is believed that the imbalance between the production of oxidants and the presence of anti-oxidants may lead to oxidative stress (Yenilmez et al., 2022). Investigating the plasma levels of Vitamin E indicates that the blood Vitamin E drops to minimum levels around calving (Mary et al., 2021) while the immune system is impaired. Vitamin E is a fat-soluble anti-oxidant that appears to be effective against oxidative stress. The results of the cows treated with 20 ml of Vitamin E indicated that it could protect them against oxidative stress (Yenilmez et al., 2022). It has been demonstrated that Vitamin E administration could reduce malondialdehyde (MDA, a biomarker of oxidative stress) concentrations 4 hours post-calving (Mokhber-Dezfouli et al., 2008). Furthermore, the risk of postpartum disorders such as retained fetal membrane and mastitis may be increased when cows suffer from Vitamin E deficiency (Qian et al., 2021). In a recent review article regarding the effects of hypovitaminosis E on transition cows, the authors strongly proposed that Vitamin E deficiency is a risk factor for the incidence of postpartum diseases (Haga et al., 2021). It is important to note that, in addition to the impact of Vitamins deficiency on both dam and offspring, as discussed above, deficiency of trace minerals such as zinc, copper, selenium, and manganese may have profound effects on maternal and offspring health and later performance (Van Emon et al., 2020).

As noted, dairy cows suffer from metabolic and immune stresses during the dry period, especially in late gestation. All nutritional and environmental factors that affect the dam might have carryover effects on the neonatal calf. In addition to metabolic and immune dysfunction, nutritional imbalance may negatively influence the fetus's development and organ function, which can have long-lasting effects on calf physiology and susceptibility to a wide range of diseases (Abuelo, 2020). Given that maternal and intrauterine conditions would affect the health and performance of the newborn calves, it could be concluded that some of the complexities that the young calves experience in the pre-weaning period can be explained by the nutrition and management of the dam in the dry period. The role of maternal nutrition and stress on neonatal calf diarrhea has been discussed recently (Alimirzaei and Nikkhah, 2022). The developing fetus may negatively respond to environmental disruptions which would be reflected in the organ defect of adults (Abuelo, 2020). For instance, placenta changes such as alterations in the placenta function would affect nutrient transport between mother and fetus resulting in altered fetal growth and organ development (Abuelo, 2020). Besides maternal nutritional conditions, undernutrition is thought to be an important factor influencing fetal growth and health (Abuelo, 2020). It is important to note that undernutrition and developmental programming are integrated because restricted nutrient intake is the main part of fetal programming. In beef cows vaccinated with the bovine diarrhea vaccine, restricting energy intake by 70% of the daily energy

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requirements in the last 40 d of gestation resulted in a lower titer of bovine diarrhea antibody in calves at 306 d of life (Moriel et al., 2016). On the other hand, adequate nutrition is needed for milking pregnant cows to avoid nutrient competition between the dam and the growing fetus (Abuelo, 2020). Inadequate nutrient intake of milk-producing pregnant animals would result in calves with poor health and low efficiency (Abuelo, 2020).

Stocking density

Providing a comfortable environment with minimum stress is a golden key in modern dairy cows' management. The transition period, as mentioned above, is considered the most critical period of the dairy cows' life cycle, needing considerable nutritional and non-nutritional considerations. The role of the environmental stressors in the prepartum cows can be considered at the maternal and neonate levels. It has been demonstrated that non-nutritional stressors (such as stocking density and grouping) could reduce the DMI of prepartum cows and increase the risk of postpartum disorders (Drackley and Cardoso, 2014). Additionally, offspring health and performance could also be affected by maternal stresses. It is believed that offspring brain development may be impaired when the mother is exposed to stressful conditions (Charil et al., 2010). From a practical outlook, some of the sources of stress for dry cows may be ignored by owners or farm managers. It is quite natural in large dairy herds that many animals give birth during the same time (a season or a month). In such herds, the prenatal animals may be overcrowded in their pens. As a result, overcrowding or higher stocking density can be a stressor and may alter their behavior and physiological status, predisposing them to various health problems. In a study conducted to investigate the laying and ruminating behavior of prepartum cows under different density rates (80, 100, and 120%), the results indicated more laying and ruminating time for the density of 80% between d -21 to d -7 relative to parturition (Jiang et al., 2021). Blood metabolites analysis also showed a higher calcium level for cows in the 80% group. Milk yield in the first month of lactation was also greater for the 80% group. In another study investigating the effects of high stocking density in late gestation on calf health, behavior, and welfare, the authors described that maternal high stocking density could affect offspring behavior in the pre-weaning period (Fujiwara et al., 2020). In this study, calves born to dams under overcrowding stress (H calves) showed increased reactivity to weaning. In addition, the H calves had decreased laying time around (just before and after) weaning. As such, the results provide evidence that calves' welfare during the pre-weaning period can be a function of maternal conditions such as stocking density. The role of maternal exercise on circulating cortisol and subsequently increased weaning stress in offspring has been recently reported (Black et al., 2017). Investigating the role of stocking density during the dry period indicated elevated levels of dehydroepiandrosterone (DHEA) and cortisol in the overstocked group relative to the control group (Fustini et al., 2017). Higher stocking density may result in limited access to feed bunk and altered animal behavior, leading to feed sorting, rumen acidosis, and reduced feed intake (Proudfoot et al., 2009). These events might induce further metabolic stresses in prepartum cows that could increase the risk of postpartum disorders. The stocking density in the prepartum period should be at least one cubicle of resting space per cow (Cook et al., 2005).

Regrouping

At the farm level, dairy cows are moved between pens according to their milk yield, days in milk (DIM), and BCS. In addition, dairy cows are transferred to dry groups following the drying off and maybe regrouped into the close-up pen until calving. Moving to new pens or places is stressful for cows because they try to reestablish their social relationships and modify physical and non-physical interactions with their new pen-mates (Huzzey et al., 2006). The common competition occurs at feed bunk where the prominent cows try to reach the feed first, which can affect feed intake (Huzzey et al., 2006). Such competition is frequently seen in higher stocking density pens; thus, overcrowding must be avoided (Huzzey et al., 2006). It seems that regrouping may induce some behavioral and physiological alterations in transferred cows (Zelena et al., 1999; Von Keyserlingk et al., 2008). It has been reported that cows spent 15 minutes less time eating following introduction to a new group (Von Keyserlingk et al., 2008). In that experiment, cows were displaced more than 25 times during the day after regrouping, whereas this value was 10 before regrouping. As such, in dry cows, regrouping has been reported to impact the feed intake of moved cows. Similar to lactating cows, a study on the effects of regrouping on dry cows' behavior showed that cows reduced their DMI by approximately 9% after moving to a new pen (Schirmann et al., 2011). The authors concluded that regrouping could affect dry cows' feeding and social behavior. It seems that cows and heifers have different responses to new environments. Heifers are more sensitive to regrouping than cows (Soonberg et al., 2021) because cows are more experienced and settle in a new environment more quickly than heifers (Soonberg et al., 2021).

CONCLUSION

Dairy cows undergo tremendous metabolic adaptations during the transition period. It is widely accepted that nutritional and management strategies for dry cows must be in line with decreased metabolic and environmental stresses to optimize fresh cow and calf health and performance. Under- or over-nutrition of dry cows as well as trace minerals and vitamins

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deficiency, may have a profound effect on both dam and offspring health and their susceptibility to metabolic and infectious diseases. As a result, providing a balanced diet is critical for the optimal health of transition cows. In addition, minimizing the environmental stressors such as stocking density and regrouping of dry cows can contribute to overcoming metabolic adaptations and immune stresses during the periparturient period.

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Data availability and materials
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Authors' contributions
The authors contributed to writing the initial manuscript almost equally. Akbar Nikkhah led the project, conceptualized the review idea, strategized the topic development, and did the ultimate writing and editing. The final manuscript was checked by all authors.

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Ethical consideration
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Effectiveness of *Solenostemma Argel* Extract on *Dermanyssus Gallinae* in Budgies (*Melopsittacus undulatus*)

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ABSTRACT

Dermanyssus gallinae (*D. gallinae*) is an important ectoparasite in veterinary and human medicine due to its role as a vector of infectious disease-causing pathogens and its economic impact. The present study reported the infestation of 45 budgies (*Melopsittacus undulatus)* reared in an aviary at the Jardin d'essai Zoo, Algiers, Algeria, showing signs of pruritus with sores due to itching. Skin samples were taken from the 45 budgies (26 females, 19 males), 26 nests, and an aviary containing the budgies were microscopically analyzed. The findings indicated the presence of *D. gallinae (*hematophagous mite*)* in all budgies and nests. To fight against this red mite, a treatment based on the *Solenostemma argel* (*S. argel*) aqueous extract was implemented. The *S. argel* aqueous extract 2% showed a fast and effective influence on *D. gallinae* mites present in the budgies, nests, and aviary. The aqueous extract of *S. argel* leaves could be a good candidate in the fight against red mites.

Keywords: Aqueous extract, Budgie, *Dermanyssus gallinae*, *Solenostemma Argel*

INTRODUCTION

Dermanyssus gallinae (D. gallinae), also called false louse or red louse of poultry, is a hematophagous avian parasitic mite with nocturnal activity. Its reservoir is usually the nests of various birds, aviaries, and chicken coops (Magdoud et al., 2019). The *D. gallinae* can infest other hosts, particularly dogs, cats, rabbits, horses, and humans (Dogramaci et al., 2010). The *D. gallinae* mite infestation is a serious public health concern, as the prevalence of red mites is expected to increase due to recent changes in hen-rearing practices, increased resistance to acaricides, global warming, and the lack of a sustainable approach to control infestations by this parasite (Sigognault Flochlay et al., 2017). The *D. gallinae* can cause great economic losses justifying the development of new effective and safe treatments for animals (Sadr et al., 2022). The increase in the rate of resistance to acaricides used against this parasite limits the effectiveness of these products (Decru et al., 2020). Several research studies have been carried out to establish alternative herbal treatments for the treatment and prophylaxis of *D. gallinae* infestation (Ghavami et al., 2020, Amer et al., 2021; Sadr et al., 2022). This clinical case falls within this context.

Solenostemma argel (*S. argel*) is a tropical plant widely used in traditional medicine for the treatment of several ailments, including gastrointestinal disorders, diabetes, rheumatism, lung, liver, and kidney infections, pain, inflammation, and wounds (Innocenti et al., 2005). The *S. argel* is distributed in the desert regions of Algeria, Egypt, Libya, and Sudan (Benmaarouf et al., 2020). The aerial parts of *S. argel* were formerly used in animal husbandry to control parasites present in animal drinking water (Benmaarouf et al., 2020). The *S. argel* extracts are known to have antibiotic, antifungal (Farrah and Ahmed, 2016), and insecticidal properties (Gipreel et al., 2020). The aim of this case study was to make known the effectiveness of the aqueous extract of *S. argel* on mite *D. gallinae* eradication.

CASE REPORT

The case of the current study was a budgie aviary located in the Jardin d'essai Zoo in Algiers, Algeria. The aviary housed 45 budgies (*Melopsittacus undulates*), consisting of 26 females and 19 males, aged between 1 to 4 years (Figure 1). The zoo care staff reported signs of pruritus with squama and sores due to feather pecking, stress, and restlessness in the birds. Furthermore, the sanitary condition of the aviary was reported to be unsanitary, with bird feces present on the floor and in the drinking water (Pavlicevic et al., 2019; Decru et al., 2020). The sanitary condition of the aviary was not safe for birds. More precisely, the aviary, floor, and drinking water were soiled with bird feces.

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The study was performed on October 2022 under a high relative temperature of 35°C and humidity of 70%. The clinical examination by a veterinarian highlighted the presence of pruritus signs as well as scales on the whole of the budgies' skin and in a greater quantity under their wings. Regarding the general clinical signs, the veterinarians observed a lack of appetite and restlessness in all individuals.

Figure 1. Budgerigar aviary in the Jardin d'essai zoo of Algiers, Algeria

Complementary examinations

To collect dander, skin samples were taken from the budgies in the form of scrapings, with at least one sample per animal. Feathers were also taken. In addition, samples were taken from the soil of the aviary and 26 nests. All the samples were then stained with lactophenol cotton blue (Sigma-Aldrich, Merck, Germany) and observed under an optical microscope (10x, Motic BA310, MoticEurope, Germany). Direct examination of collected specimens at the veterinary school of Algiers, Algeria, revealed the presence of blood-engorged *D. gallinae* mite (Figure 2 A), characterized by styliform chelicerae, long legs, and a dorsal shield narrowing at the rear (Sparagano and Giangaspero, 2011). Direct microscopic examination also indicated *Malassezia spp*. yeasts in very large numbers per field (Figure 2 B). The presence of *Malassezia* yeasts was a sign of a fungal superinfection, probably due to the weakening of the immune defenses (Velegraki, 2015; Hobi et al., 2022).

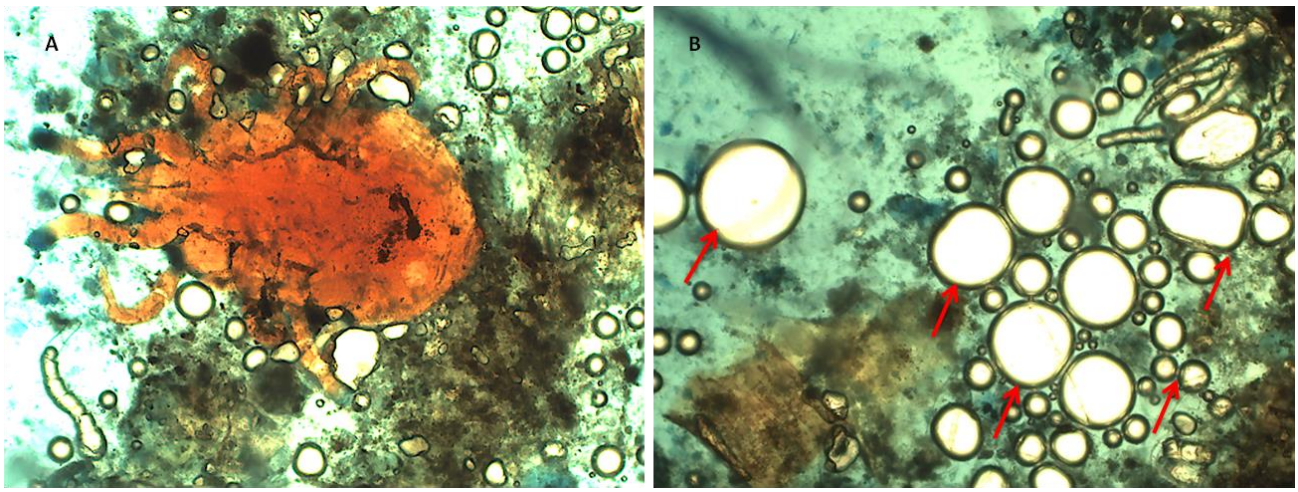

Figure 2. *Dermanyssus gallinae* **(A)** and *Malassezia spp*. (**B,** white spherical structures arrowed) collected from *Melopsittacus undulatus* (Algiers, Algeria, October 2022), magnification 10x.

Preparation of the extract

The aqueous extract was prepared from the leaves of the *S. argel* plant harvested in southern Algeria in the region of Abalessa in Tamenrasset in February 2022. The identification of the plant was carried out by the botanical service of the National School of Agronomy (Ecole Nationale Supérieure d'Agronomie, ENSA) of Algiers in Algeria with the delivery of an identification certificate (ENSA 08/01/2020). Reference specimens have been deposited in the herbarium

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of the Animal Health and Production Laboratory of the National Veterinary School of Algiers (Santé Production Animale, SPA. 031).

The lyophilized aqueous extract was prepared by mixing 20 g of powdered dried leaves of *S. argel* in 1000 ml of boiling water, and the mixture was boiled in a water bath for 30 min. The mixture was filtered and then adjusted to 1000 ml before being lyophilized (Abubakar and Haque, 2020). To make the 2% aqueous solution, 20 g of the powder was dissolved into 1 liter of sterile distilled water (Unpublished data).

Treatment

In order to eliminate the blood-sucking mite *D. gallinae*, the budgies were individually sprayed on the whole body and especially below the wings using a sprayer with a 2% aqueous solution based on *S. argel* for a week. The aviary and the nests were disinfected with the 2% aqueous extract of *S. argel*. Each day after the treatment, the budgies were individually examined by a veterinarian for the presence of squama and itching. A marked improvement was noticed from the first application. On day 7, one skin sample (scratching) per animal below the left wing was taken from the budgies and from the aviary and nests to verify the effectiveness of the treatment. Direct examination of the skin samples under an optical microscope (10x, Motic BA310, MoticEurope, Germany) revealed the absence of *D. gallinae* on all of the examined samples from the budgies (*Melopsittacus undulatus*, Figure 3 A, B, C) and in the aviary and the nests (Figure 3 D). In addition, interestingly, the commensal *Malassezia* yeasts were found in smaller numbers on budgie samples (Figure 3 C).

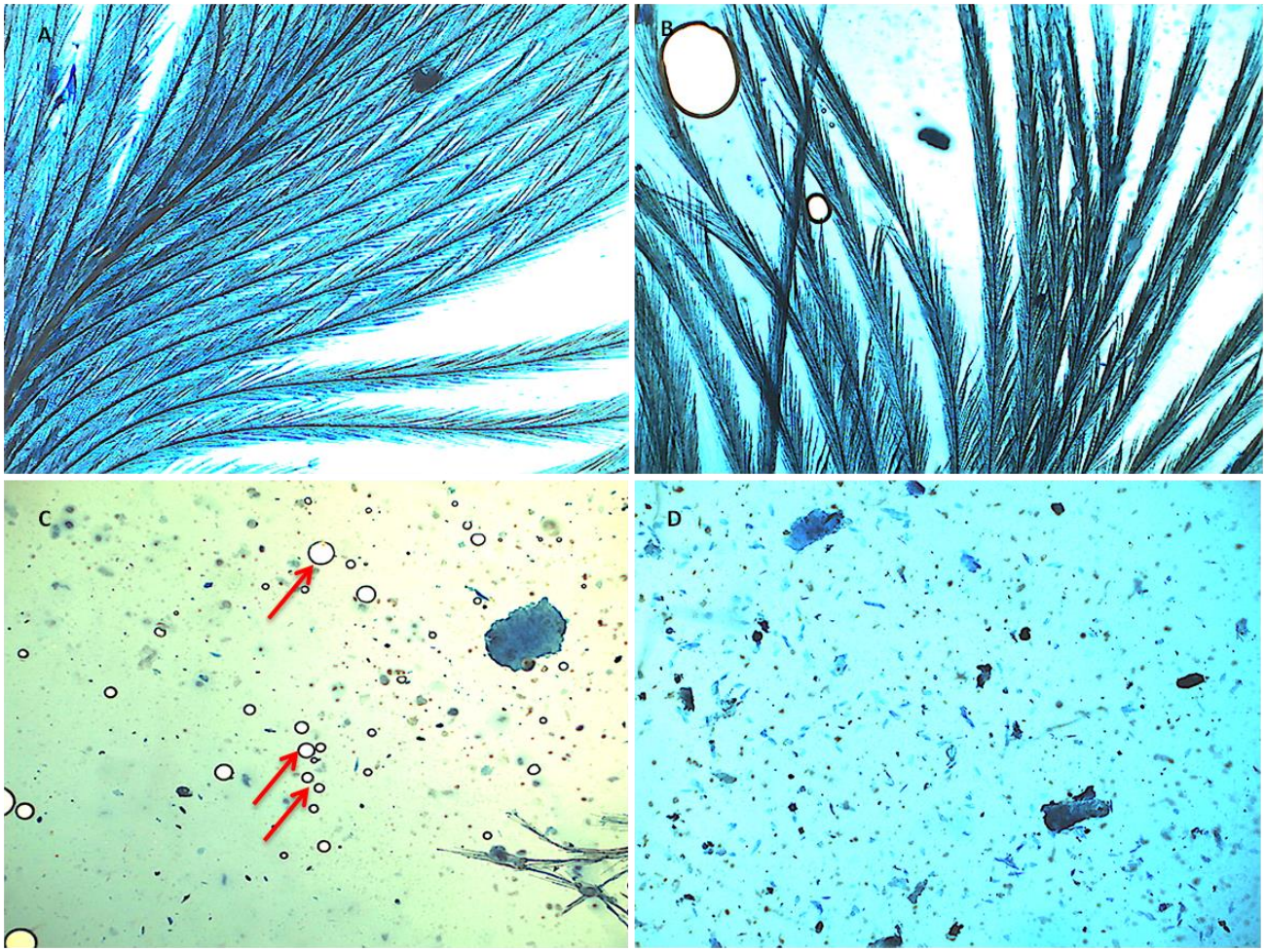

Figure 3. The treatment effects of aqueous extract of *S. argel* on *Dermanyssus gallinae* in samples with different sources in a zoo of Algiers (Algeria). **A, B, C:** Skin samples from budgies ×4, **D:** Samples from aviary and nests x4. Red arrow: *Malassezia* spp. (spherical structures).

DISCUSSION

The *D. gallinae* is a cosmopolitan species, the most common Dermanyssidae mite, which parasites a wide range of hosts (Mullen and Oconnor, 2019). This mite is a major threat to the poultry industry and the breeding of ornamental birds worldwide, causing economic losses and serious animal health problems (Decru et al., 2020). The *D. gallinae* is a parasite of major importance both in veterinary and in human medicines insofar as it plays a vector role for several

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pathogens, in particular, the bacteria *Escherichia coli*, *Pasteurella multicida*, *Coxiella burnetii*, *Erysipelothrix rhusiopathiae*, *Borrelia burgdorferi*, and *Salmonella enteritidis* as well as Avian Influenza virus A, Newcastle virus and equine encephalomyelitis virus (Valiente et al., 2007; Sparagano and Giangaspero, 2011; George et al., 2015; Sommer et al., 2016; Sigognault Flochlay et al., 2017).

The increasing prevalence of *D. gallinae* mite infestation around the world is a matter for concern, and can be attributed to several factors. These include the transformation of housing systems into avian breeding facilities, non-compliance with proper aviary management practices, global warming, and a lack of effective methods for controlling infestations, as well as the increasing resistance of mites to commonly used acaricides such as carbamates and pyrethroids (Sparagano and Giangaspero, 2011; Sigognault Flochlay et al., 2017). Furthermore, a shortage of effective acaricides has been reported, as several chemical treatments have been withdrawn from the market due to their adverse effects on humans, which limits the available options for controlling mite infestations (Abbas et al., 2014; Sigognault Flochlay et al., 2017).

In Europe, very few products are licensed for use against *D. gallinae*, and except for a recently approved phoxim-based product, they can be used only when the poultry house is empty, that is, between two productive cycles (Sparagano and Giangaspero, 2011).

The present clinical case demonstrated the efficacy of *S. argel* aqueous extract on budgies *M. undulatus* infested with the mite *D. gallinae*. The extract also allowed the elimination of the red mite from the nests and the aviary. Several studies have highlighted the phytochemical composition of *S. argel* (Tigani and Ahmed, 2009; Shafek and Michael, 2012; Benmaarouf et al., 2020). In particular, the main constituents of the *S. argel* leaves extracts are flavonoids (rutin, quercetin, kaempferol, Cen-Pacheco et al., 2020), alkaloids (Wu et al., 2021), tannins (Fernández-Salas et al., 2011) and saponins (Pavela, 2016; Teia, 2018) known to have antibiotic and acaricide effects. Therefore, *S. argel* extracts represent an interesting candidate in the fight against the hematophagous mite *D.gallinae*. The insecticidal effect of *S. argel* extracts was previously described on *Tribolium castaneum* (Gipreel et al., 2020) *or Culex pipiens* (Al-Mekhlafi et al., 2018). Several recent studies have highlighted the acaricidal effects of plant-based products against *D. gallinae*. The studies carried out previously showed the effectiveness of 50 plant essential oils (such as garlic, thymus, or tea tree) on red mites with mortality rates ranging from 80 to 100% (George et al., 2009; George et al., 2010). Another study demonstrated significant acaricidal activity of the essential oils of cade, clove, mustard, laurel, coriander, pennyroyal, red and white thyme, cinnamon, and spearmint against *D, gallinae* using direct contact and fumigation methods (Kim et al., 2004).

However, favorable sanitary conditions are necessary to guarantee the effectiveness of different drugs and extracts in control of the red mite. the aqueous extract of *S. argel* leaves seems to have an effect on the reduction of *Malassezia* yeasts, indicating an antifungal effect. It is in agreement with a previous study showing an antifungal effect on *Candida albicans* (Farah and Ahmed, 2016).

CONCLUSION

The aqueous extract of *S. argel* allowed total eradication of the blood-sucking mite and zoonotic agent *D. gallinae* in the current study. The aqueous solution of *S. argel*, therefore, could be an effective and safe means of combating this ectoparasite. Moreover, the antifungal effect of *S. argel* extract is also suggested. The effect of *S. argel* extract on different ectoparasites, as well as the determination of the active substances, can be considered in future studies.

DECLARATION

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Availability of data and materials

The raw data are available on demand from the corresponding author.

Authors' contributions

Daouia Keltoum Benmaarouf (DKB) is the major investigator, Amina Laieb is the zoo veterinarian collaborating with DKB for the diagnosis and the treatment of the animals, Bernard China was active in the redaction and verification

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of the manuscript, Nezha Khouchane is the director of the Zoo allowing the study, Meriem Ben Mahdi is the research supervisor. All authors confirmed the final draft of the article for submission to the journal.

Competing interests

The authors declare no conflict of interest.

Ethical consideration

The authors take steps to abide by all ethical standards related to plagiarism, publication approval, inaccuracies in data, multiple submissions, and double publication.

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The Protective Effects of Melatonin against Brain Disorders Induced by the Western Diet in Male Rats

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ABSTRACT

Globally, the effects of consuming a diet rich in fat have gained great concerted attention. The current study was conducted to evaluate the protective effects of melatonin on neurological disorders induced by the western diet in rats. A total of 30 adult male white local Iraqi rats were randomly assigned to three equal groups, including control (CC), high-fat diet (HFD), and melatonin group (HFD+M, a high-fat diet along with intraperitoneal injections of 10 mg/kg body weight melatonin) for 8 weeks. The rats were analyzed in terms of brain tissue concentration of dopamine, tumor necrosis factor (TNF), and nervous system impairment using Barns maze task and elevated plus maze. The findings revealed a significant decline in the dopamine concentration of the HFD group after 8 weeks of treatment, compared to CC and HFD+M groups. Moreover, there was a significant increase in brain TNF-α concentration in the group fed HFD, compared with CC and HFD+M. Finally, the melatonin treatment significantly reduced spatial memory impairments and anxiety induced by HFD in rats. After 8 weeks, the histological examination revealed that brain section rats on an HFD indicated significant congestion in the blood vessels with marked cerebral edema, where there was a dilation of Virchow-Robin space, severe congestion, and infiltration of inflammatory cells in the meninges. The HFD+M groups showed normal meninges without any inflammatory exudate except for a few congestions in the blood vessels and no or mild vacuolations in the cerebral tissue, gliosis, and astrocytosis. In contrast, male rats fed an HFD showed vacuolation and aerophagia in brain tissue and a marked aggregation of the proliferation of astrocytes and a proliferation of microglial cells in the cerebral. In conclusion, HFD impairs brain neurotransmitters, induces pro-inflammatory changes, and affects learning ability and memory by changing the structure of neural tissue. Melatonin can ameliorate HFD-caused effects.

Keywords: Brain, High-fat diet, Inflammatory cells, Learning ability, Melatonin, Nervous system

INTRODUCTION

The earliest definition of a high-fat diet (HFD) as a nutritional strategy to enhance obesity was published in 1959 (Mašek and Fabry, 1959). The HFD can be well observed in the Western, high-energy, high-fat, cafeteria, and high-fat sugar diets. The precise nutrition structure of the control and fat diets, including the quantity and types of carbohydrates of fats, may vary and is not extensively detailed (Mozaffarian et al., 2011; Mota et al., 2023). It is unknown whether strains, ages, and species of animals may affect the outcomes of consuming HFD experiments or whether adjusting critical parameters, such as weight, behaviors, and memory, may be affected by the type and duration of diet exposure (Abbott et al., 2019). Obesogenic feed often contains 60% of total calories as fat, compared to 30-40% fat in a typical Western diet (Lai et al., 2014).

During lipopolysaccharide-induced end toxemia, the spleen releases newly-produced tumor necrosis factor-alpha (TNF) into the liver. The primary source of TNF is end toxemia, which is released from the liver and circulates throughout the body (Tracey, 2007). Tumor necrosis factor-alpha is a critical cytokine with different harmful effects, including the production of more inflammatory cytokines with the infiltration of macrophages (Tracey, 2007). The HFD can enhance the oxidative stress, inflammation, and activation of Nuclear Factor kappa Beta-cell (NF-kB) in the rat cerebral cortex, raising the possibility that HFD increases dementia risk (Zhang et al., 2022). Motivation, reward, punishment, energy expenditure, and working memory are all functions of dopamine, which has been recognized as an important neurotransmitter in brain function (Cools, 2008). Increased dietary fat intake has been linked to a decrease in dopamine signaling, which may increase the calorie intake to compensate for this decreased dopamine. Dopamine is a neurotransmitter that influences food intake, particularly pleasing dietary ingredients. However, increased dietary fat intake has been linked to a decrease in dopamine signaling, which may increase the calorie intake to compensate for this decreased dopamine (Vucetic and Reyes, 2010; Hryhorczuk et al., 2016; Joshi et al., 2021). Studies have confirmed that the brain is sensitive to dietary essential fatty acids, and such a diet can make remarkable changes in membrane composition, consequently altering neurons' metabolic

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properties. In other words, changes in dietary fat composition could have a significant effect on membrane composition and neuronal functions of the brain (Dyer and Greenwood, 1988; Adermark et al., 2021; Dyall et al., 2022).

The central nervous system consists of several distinct brain areas that control memory and learning processes; however, the hippocampus has a prominent function. The dorsal hippocampus appears to be primarily linked to cognition, while emotion, affect, and stress bind in the ventral hippocampus; this region is unique in that its anatomical activities are divided along the dorsoventral axis (Fanselow and Dong, 2010). Both the dorsal and ventral gyrus of the hippocampus are collectively referred to as the dentate gyrus and are sites of postnatal hippocampal neurogenesis. This process facilitates the maturation of new neurons, which eventually become integrated into the hippocampal circuitry and contribute to its function. The dorsal hippocampus, in particular, plays a crucial role in spatial memory processing (Bannerman et al., 2014; Bortolotto et al., 2014).

There may be a benefit to the Barnes Maze over the Morris Water Maze for subjected rodents which have swimming difficulty because of obesity or other metabolic problems brought on by an HFD (Pitts, 2018). The elevated plus maze test assesses anxiety in lab animals. As a general research instrument, a maze test is typically conducted on rodents for neurobiological anxiety studies and acts as a screening test for potential anxiolytic or anxiogenic substances (Kraeuter et al., 2019). The western diet (rich in fat and sugar) has also been linked to learning, memory (spatial), cognition, and hedonics (Francis and Stevenson, 2013). Spatial memory loss and cell death in the hippocampus can be caused by fat-rich meals (Asadbegi et al., 2017). Moreover, the unbalanced production of reactive oxygen species and the body's antioxidants play a significant role in the neurotoxicity caused by an HFD. When exposed to oxidative stress, cell death occurs due to hydroxyl radical formation, lipid peroxidation, and apoptosis (Ganji et al., 2017; Rozha et al., 2022). Studies have shown that a diet mainly containing saturated fatty acids with trans-fatty acids increases the melatonin hormone secreted from the pineal gland during the dark period and has a vital role in immunity. Daily melatonin administration affects some physiological parameters, including glycemic index, leptin, and dopamine (Al-Azawi et al., 2003). In addition, melatonin decreases lipid levels by increasing the conversion of endogenous cholesterol to bile acids and suppresses cholesterol synthesis and accumulation (Kara and Kara, 2022). Melatonin stimulates the synthesis of antioxidant enzymes, including superoxide dismutase, glutathione peroxidase, and glutathione reductase (Sabeeh and Khudair, 2017).

Melatonin N-acetyl 5methoxytryptamin, isolated for the first time from pineal glands of bovine (Lerner et al., 1958; Venegas et al., 2012), is an endo-neurohormone derived from tryptophan (García-Bernal et al., 2021). The lack of melatonin can lead to various health problems, including neurodegenerative illnesses, circadian rhythm and mood disorders deprivation, diabetes type two, and pain (Comai and Gobbi, 2014). Some health problems, such as obesity, diabetes, hypertension, and respiratory diseases, can be linked to sleep deprivation (Kuvat et al., 2020) since sleep deprivation negatively affects biological and physiological processes (McEwen, 2006; Yin et al., 2017). Melatonin secretion occurs just as sleep propensity since there is a decrease in core body temperature, alertness, and performance (Pandi-Perumal et al., 2008; Borbély et al., 2016). Hippocampal neurons directly respond to melatonin's effects on memory formation (Chang et al., 2021). Melatonin has been shown to have anti-nociceptive, antidepressant, anxiolytic, anti-neophobic, and locomotor activity-regulating effects (Uz et al., 2005; Mantovani et al., 2006; Fenton-Navarro et al., 2021). Melatonin can mediate the effects of mitochondria on physiological processes (Reiter et al., 2014). Melatonin improves the flow of electrons in the inner mitochondrial membrane, which in turn protects the morphology of the cell membrane. It also boosts the activity of antioxidant enzymes, scavenges free radicals, and enhances functional aspects of the cell (García et al., 2020). Improved membrane fluidity, decreased edema, and reduced infiltration of polymorphonuclear cells into damaged tissue are among the factors that result in a decrease in the expression of pro-inflammatory cytokines. Melatonin achieves this by preventing nuclear factor-kB translocation to the nucleus and binding to DNA, both of which are crucial factors in the inflammatory response (Mayo et al., 2005). Methamphetamine results in an expression reduction of dopamine transporter in the rat hippocampus, which was stopped and reversed by melatonin (Panmak et al., 2021). With this in mind, the current study aimed to examine the detrimental effects of a high-fat diet on animal behaviors and brain processes and to investigate the potential of melatonin to mitigate any negative effects.

MATERIALS AND METHODS

Ethical approval

Animal care and treatment in this study were carried out at the Faculty of Veterinary Medicine, University of Baghdad, Iraq, strictly following the code of ethics for animal experiments (P.G. 201 date 25-1-2023).

Study design

A total of 30 adult male white local rats with an average weight of 160-200 g were housed in special plastic cages (15×70×60 cm) in a room at the animal house of the Veterinary Medicine College, Baghdad University, Iraq. The rats were sustained under controlled conditions for 12 hours of light and 12 hours of darkness at the temperature of 22-25°C

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and 40% ± 5% relative humidity. The rats were acclimatized for 10 days prior to the trial. For adaptation, food and water were provided *ad libitum.* The study lasted 8 weeks. The experimental rats were divided randomly into three equal groups (10 rats in each group). The first group was the control group (CC), in which the rats were fed a basal diet throughout the experiment and received an intraperitoneal (IP) injection of normal saline (Table 1). The second group was fed an HFD for 8 weeks with an IP injection of normal saline during the experiment (Table 1). The third group received the HFD plus melatonin (HFD+M), so the rats were fed a saturated HFD for 8 weeks with an IP injection of 10 mg/kg/daily body weight melatonin (Taher and Arrak, 2016; Maher et al., 2020).

Preparation of the high-fat diet

The diet rich in fat used in the current study was made weekly and stored in sealed bags, kept out of light, and stored at 4°C and humidity less than 50% until used in pelleted form or as round. The HFD formulation and composition are shown in Table 1.

Table 1. Diet ingredients in the present study

Ingredient	High-fat diet (g/kg diet)	Normal diet (g/kg diet)
Corn starch	150	650
Casein	200	200
Tallow fat	400	0
Oil	0	50
Sucrose	40	40
Cellules	50	50
Vitamin mix	10	10
Methionine	3	3
Salt	2	2

Source: Mashmoul et al. (2017).

Tissue collection and homogenization

At the end of the 8-week experiment, all rats from all groups were anesthetized with intramuscular injection of Ketamine (60 mg/kg) and Xylazine, and subsequently decapitated to remove their entire brain. The brain was dissected and divided into the left and right. To conduct the ELISA test, the right part of the brain was put in 1 ml of cold phosphate buffer saline solution containing 0.5 grams of the right part of the brain. Afterwards, the mixture was homogenized using an electrical homogenizer on ice, tissue homogenates were then centrifuged at 5000 rpm for 5 minutes, and an aliquot of the supernatant was isolated to determine TNF-α and dopamine 40 µl from an aliquot for each parameter. The left part of the brain was kept in plastic containers with formalin (10%) for routine histological examination.

Parameters

Dopamine and TNFα concentration in a male rat brain (ng/L) were measured using ELISA kit (Bioassay Laboratory Technology, Chain). Nervous system impairment was assessed using Barns maze and Elevated plus maze. The Barnes Maze comprises a dry, raised circular platform with many potential escape holes around its perimeter. A secret escape box is concealed behind a single hole. The Barnes labyrinth was effective for testing spatial memory. The typical Barnes maze protocol includes three phases, including habituation, earning (training), and memory. To quantify rat anxiety, Elevated plus maze was used. It is a plus-shaped solid white apparatus with two arms of 84cm long and 10 cm wide. Opposite arms had sides that were 2cm high (open arms), and the sides of the other opposite arms were 17 cm tall (closed arms), elevated above the floor in high 80 cm from the floor.

Histopathological study

Immediately after animal sacrifice, brains from each group were preserved in 10% formalin, and the formalin level was 10:1 of the specimen's volume. The tissues were rinsed in tap water to remove the formalin solution from the samples for three or four hours. The samples underwent various processes to make the tissue suitable for histopathological examination. These steps include dehydration, clearing, embedding, blocking, cutting, and staining (Bancroft et al., 2013).

Statistical analysis

The collected data were analyzed statistically using the Two-way and One-way ANOVA in GraphPad Prism 9.1.0. Data were subjected to a Tukey's post hoc test in case the ANOVA was statistically significant. Results were represented as mean ± standard error, with p value less than 0.05 as statistically significant.

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RESULTS

The level of dopamine

After 8 weeks of treatment, the obtained data confirmed a statistically significant decrease ($p < 0.05$) in the dopamine concentration of the HFD group, compared with CC and HFD+M groups (Table 2). However, there was a significant increase in dopamine concentration in CC and HFD+M groups, compared to the HFD ($p < 0.05$). Melatonin could elevate dopamine influenced by an HFD and increase the concentration of dopamine, when compared with the HFD group.

Brain tissue level of tumor necrosis factor-alpha concentration cytokine (ng/L)

The findings indicated a significant increase in brain TNF-α concentration group fed HFD, compared with CC and HFD+M groups ($p < 0.05$). Moreover, there was a significant increase in TNF-α brain level in the HFD+M group, compared to the control group ($p < 0.05$). However, the increment was slight and the value of tumor necrosis factor-alpha concentration (TNF2-α) for the HFD+M group was close to that of the control group. There was a significant decline in TNF-α concentration in the HFD+M (223.9±1.041), compared to the HFD group ($p < 0.05$, Table 3). As can be seen in Table 4, there was a non-significant difference between the HFD+M group and the control group at the end of the study ($p > 0.05$). However, there was a significant difference between the HFD and control groups at the end of study ($p < 0.05$).

Assessment of spatial memory using Barnes maze task

The results indicated that the HFD had a significant negative impact on spatial memory performance ($p < 0.05$). This was demonstrated by an increased time taken by the HFD group to reach the escape box at 8 weeks (5.533 ± 0.020), compared to both other groups at 8 weeks and the same HFD group at the beginning of the treatment. However, the HFD+M group demonstrated a significantly shorter time to reach the escape box (3.872±0.020), compared to the HFD group at 8 weeks. This suggests that rats fed an HFD may have a deficit in spatial memory.

Determination of anxiety by elevated plus maze

The results of this Elevated Plus maze showed the HFD was more anxious than rats in the HFD+M and CC groups. The HFD rats spent a significantly longer time ($p < 0.05$) in the close arm (4.525±. 287), compared to the control (2.863 ± 0.157) and HFD+M (3.013 ± 0.147) groups in the same arm. However, the HFD+M group spent significantly less time in close arm than the HFD group ($p < 0.05$). The HFD rats spent significantly less time ($p < 0.05$) in the open arm (1.475 ± 0.287), compared to that of the control group (3.138 ± 0.157) and HFD+M groups (2.988 ± 0.147). This means that HFD rats were highly anxious, as they spent a longer time in the close arm, compared with the other groups (Table 5).

Table 2. Effect of high-fat diet and melatonin on dopamine concentration (ng/L) in brain tissue of adult male rats

Time \ Groups	Control	High-fat diet group	Melatonin plus a high-fat diet
8 Weeks	11.80 ± 0.23^{a}	2.36 ± 0.11^{c}	6.09 ± 0.19^{b}

Data represented as mean ± standard error mean, n = 10 for each group. ^{ab}Different superscript letters in the same row indicate significant differences ($p < 0.05$)

Table 3. Effect of high-fat diet and melatonin on tumor necrosis factor-alpha concentration (ng/L) in brain tissue of adult male rats

Time \ Groups	Control	High-fat diet group	Melatonin plus a high-fat diet
8 Weeks	213.7 ± 1.249^{c}	646.1 ± 1.098^{a}	223.9 ± 1.041^{b}

Data represented as mean ± standard error mean, n = 10 for each group; ^{abc}Different superscript letters indicate the significant differences in rows between groups ($p < 0.05$)

Table 4. The effect of melatonin and high-fat diet on spatial memory barns maze test

Time \ Groups	Control	High-fat diet group	Melatonin plus a high-fat diet
Beginning of study	3.765 ± 0.028^{Aa}	3.755 ± 0.016^{Aa}	3.783 ± 0.009^{Aa}
8 Weeks	3.877 ± 0.007^{Aa}	5.533 ± 0.020^{Bb}	3.872 ± 0.020^{Aa}

Data represented as mean ± standard error, n = 10 each group; ^{ab}Small superscript letters in the same column indicate significant differences between 8 weeks and beginning time ($p < 0.05$); ^{AB}Capital superscript letters in the same row indicate significant differences ($p < 0.05$).

Table 5. The effect of melatonin and high-fat diet on anxiolytic test by Elevated Plus maze

Time \ Groups	Control	High-fat diet group	Melatonin plus a high-fat diet
Open	3.138 ± 0.157^{Aa}	1.475 ± 0.287^{Ab}	2.988 ± 0.147^{Aa}
Close	2.863 ± 0.157^{Aa}	4.525 ± 0.287^{Bb}	3.013 ± 0.147^{Aa}

^{AB}Capital superscript letters denote the significant differences in the same column ($p < 0.05$); ^{ab}Lower superscript letters indicate significant differences in the same row ($p < 0.05$); Data represented as mean ± standard error, n = 10 pre-group.

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The recent histopathological examination indicated the detrimental effect of a high-fat diet on the brain tissues of rats, and also highlighted the antioxidant and anti-inflammatory properties of melatonin, which played a role in mitigating these effects. In the hippocampus of the CC group, brain sections showed all three layers the molecular layer (ML), the middle layer (pyramidal cell layer, PL), and the inner layer (polymorphic cell layer, PCL). A normal dentate gyrus consists of three layers, including the outer molecular layer, middle granular layer, and inner polymorphic layer. The blood vessels in the molecular and polymorphic layer of the dentate gyrus were normal (Figure 1). Moreover, normal thin meninge, normal brain tissue with normal blood vessels, normal glia cells, astrocytes with oligodendrocytes, and the neuronal cell showed normal triangular perikaryon with prominent central nuclei with apical dendrites (Figures 2 and 3).

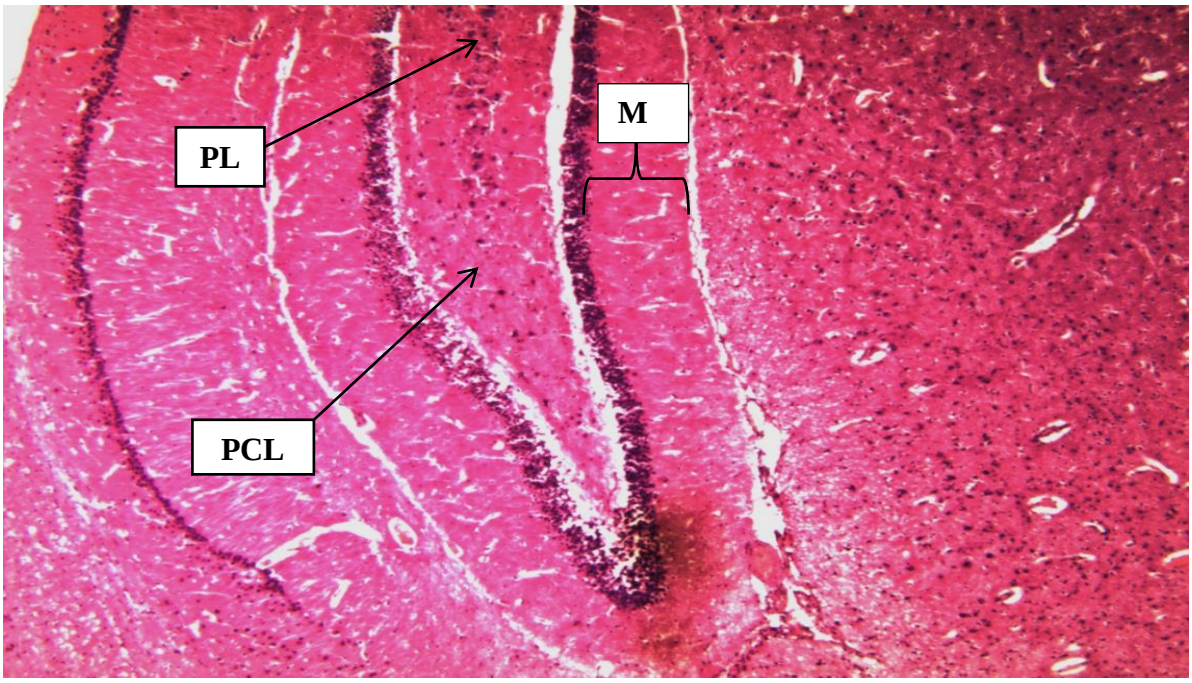

Figure 1. The brain tissue of a rat in control group. All layers of the hippocampus contain the outer layer molecular layer (ML), Middle layer pyramidal cell layer (PL), and inner layer polymorphic cell layer (PCL) are shown. There is normal dentate gyrus which contains three layers outer molecular layer, middle granular layer, and inner polymorphic layer. Normal blood vessels in the molecular and polymorphic layer of dentate gyrus. 20X (H&E).

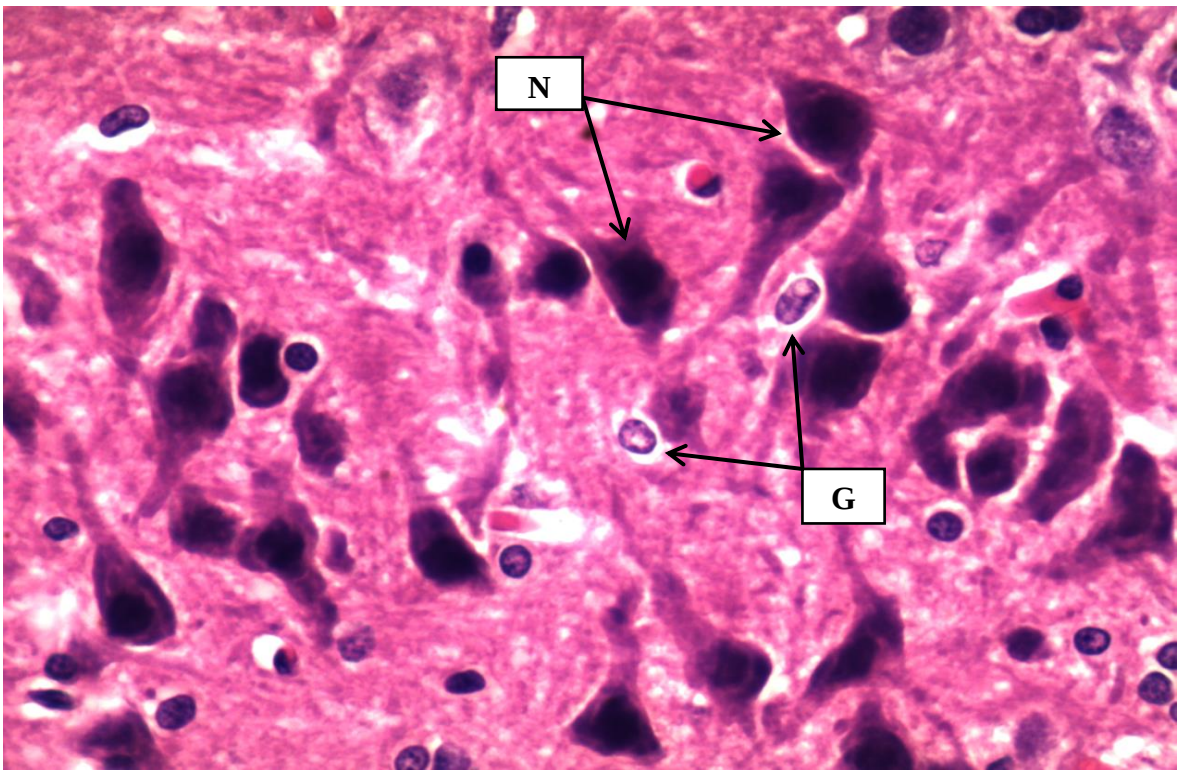

Figure 2. The brain tissue of a rat in control group. The neuronal cell (N) showed normal triangular perikaryon with prominent central nuclei with apical dendrites, and normal glial cell (G) is also seen. 200X (H&E).

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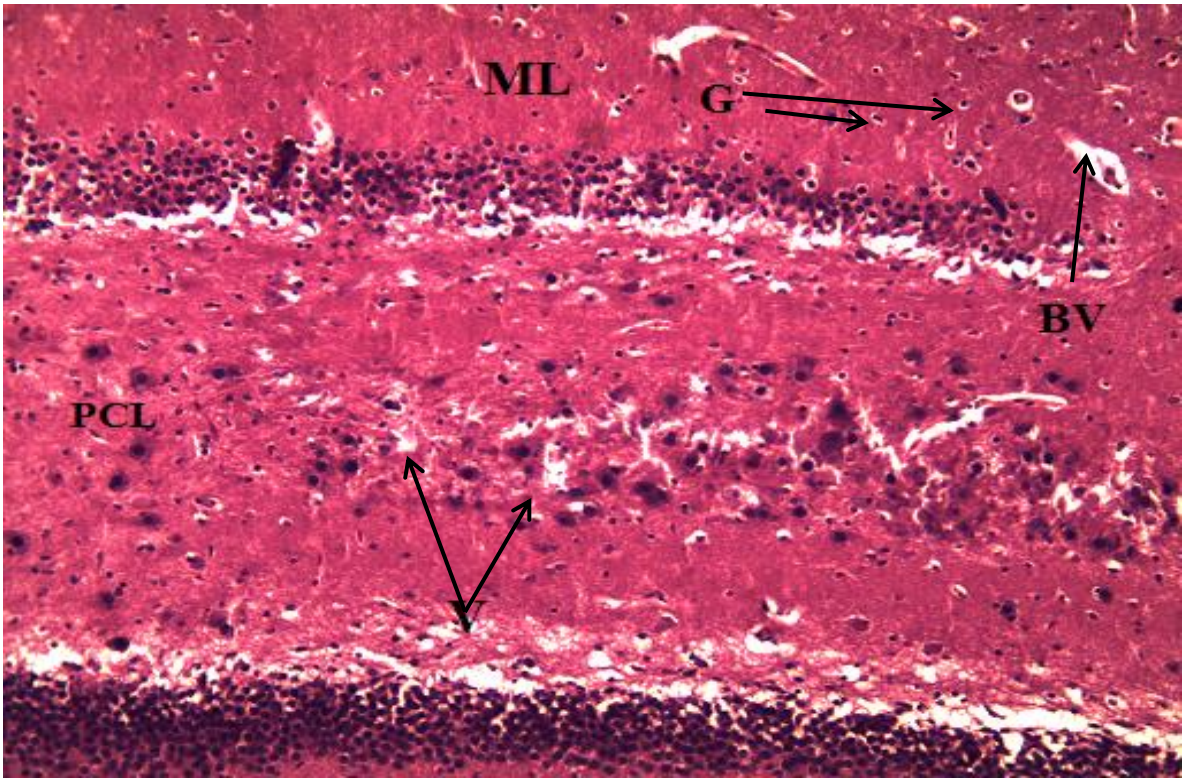

Figure 3. The brain tissue of a rat in the high-fat diet group. There is disorganization and degenerated pyramidal cells; these cells showed vacuolation (V) in the cytoplasm and pyknotic (condensed) nucleus. Dilation of blood capillaries (BV) and increased numbers of microglia (gliosis, G) in the molecular layer (ML) and polymorphic cell layer (PCL). 50X (H&E).

However, rat brain sections in the HFD group indicated disorganization and degenerated pyramidal cells. These cells showed vacuolation in the cytoplasm and pyknotic (condensed) nucleus, dilation of blood capillaries, and increased numbers of microglia (gliosis in ML and PCL), as in Figure 4. Figure 5 shows a thin pyramidal cell layer in the Cornu Ammonis with degeneration of neurons and V in the last area of cornu Ammonis and dentate gyrus at the brain of the HFD group. In addition, fine capillaries had congestion with cerebral edema in the inner polymorphic layer. In the same group, the histopathological change revealed vacuolation in the granular layer of the dentate gyrus with many glia cells (glia cells in ML and PCL of dentate gyrus), as in Figure 6. Higher magnification of the granular layer of the D indicated the marked vacuolation of the granular cells, which appear swelling, and vacuoles in their cytoplasm and pyknotic nuclei look (Figure 7).

Regarding the brain of HFD rats, the histopathological examination revealed congestion in the blood vessels with marked cerebral edema in which Virchow-Robin space dilated. Many neurological cells appeared shrunken and deeply stained, and infiltration of a high number of glia cells and Astrocytes Astrocytosis (Figure 8). In higher magnification, the neurological cell (neuron) indicated marked central chromatolysis in which the body of the neuron (perikayo) was spherical with nuclei disappearance. Moreover, gliosis and Astrocytosis were detected in the brain tissue (Figure 9). Representative photomicrographs were obtained using 40 × (200x with a factor of a camera, magnification objective). The brain rats in the HFD group indicated gliosis and Astrocytosis with the presence of Oligodendrocytes, marked central chromatolysis in the body of neurons (spherical and disappeared nuclei). Also, fine capillaries were congested in the brain tissue (Figure 10). The microscopic brain examination of rats in the HFD group can be seen in Figure 11. As indicated, there were marked degenerative areas and necrosis of neurons. The neuron showed pale color and disappearance of nuclei with marked aerophagia in the brain tissue. Cerebral edema and congestion of blood vessels were also seen. Moreover, at the same area but at a higher magnification, severe central Chromatolysis of perikaryo of neurons with gliosis and astrocytosis could be noticed (Figure 12). As can be seen in Figure 13, the brain tissue sections also exhibited severe congestion and infiltration of inflammatory cells in the meninges, vacuolation, and aerophagia and marked gliosis and astrocytosis.

According to Figure 14, most neuronal cells (neuron) appeared normal triangle perikaryo with central and prominent nuclei; however, a few of these cells had central chromatolysis with blood vessels. The histopathological section of the hippocampus in rats given melatonin was nearly similar to that of the control. The first area of Cornu Ammonis appears clear and thick, with a high number of pyramidal cells in the middle. There was a normal outer ML; however, the PCL showed few congestions in the fine capillaries. There were normal and high numbers with the normal shape of neurological cells in the last area of Cornu Ammonis, the thick dentate gyrus layer in which there were a high number and proliferation of granular cells with few vacuolations (Figure 15). Moreover, most neurons appeared normal

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with central nuclei in their perikaryo, but a few neurons underwent chromatolysis in the last area of Cornu Ammonis. There was little vacuolation in the dentate gyrus at the granular layer, mild perivascular edema in the molecular layer, and few glia cells in ML and PCL of the dentate gyrus (Figure 16).

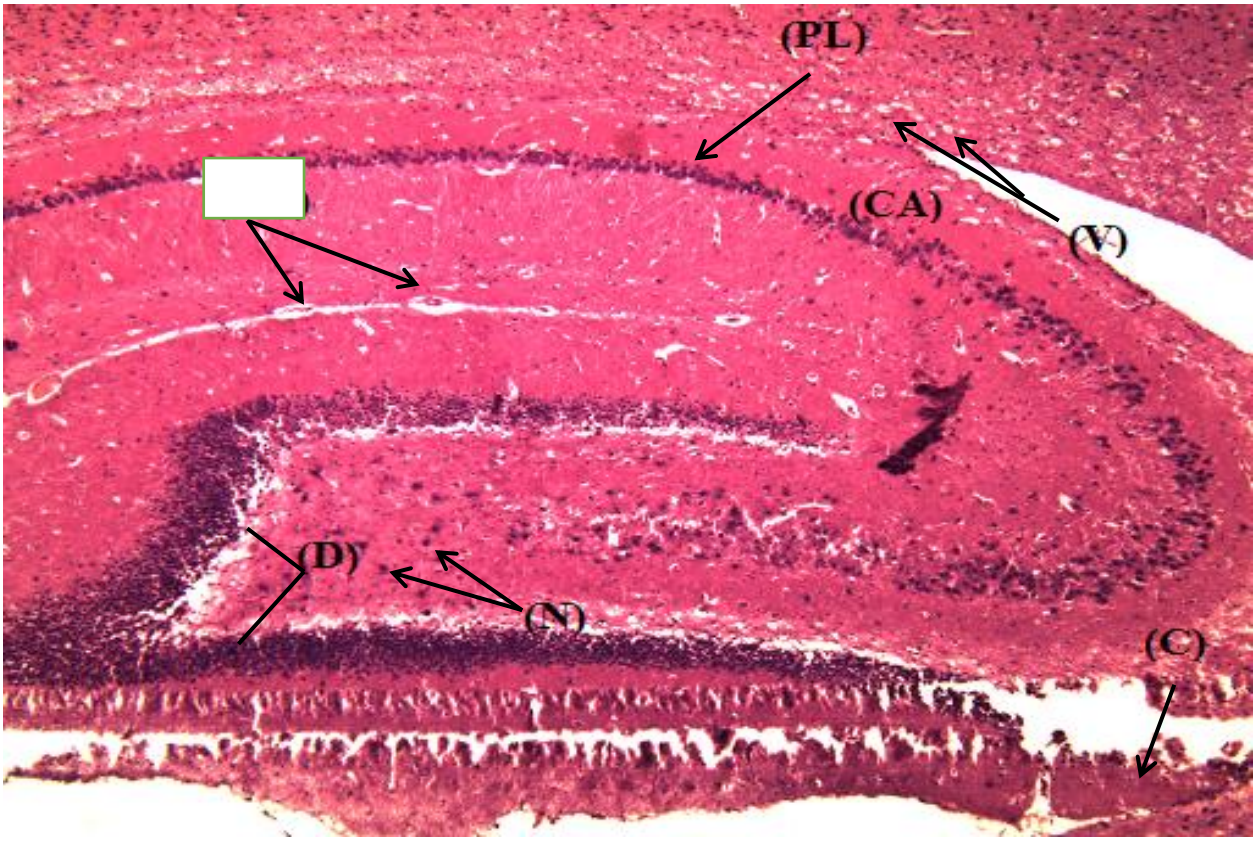

Figure 4. The brain tissue of a rat in the high-fat diet group. The thin pyramidal cell layer in the Crnu Ammonis (CA) with degeneration of neurons (N) and vacuolation (V) in the last area of Cornu Ammonis and dentate gyrus (D) are shown. There is congestion of fine capillaries (C) with cerebral edema (OD) in the inner polymorphic layer. 50X (H&E).

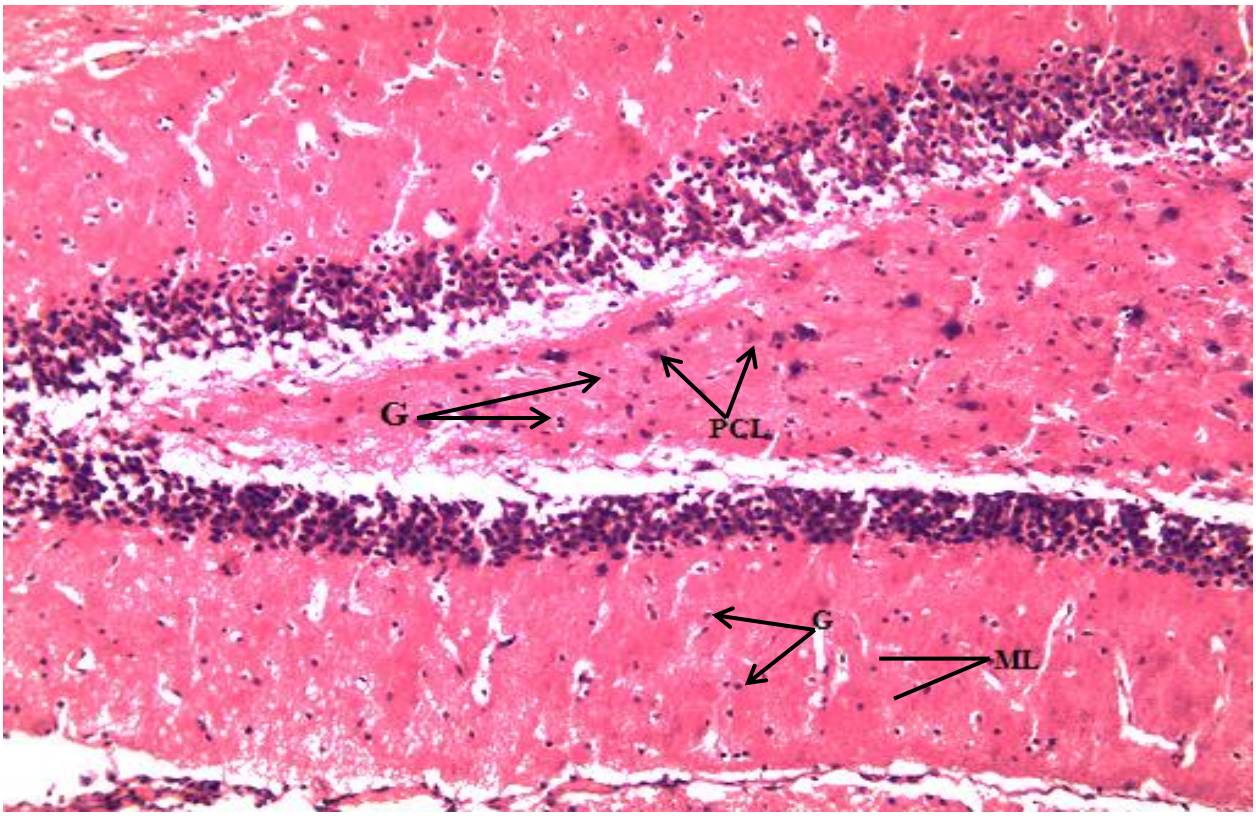

Figure 5. The brain tissue of a rat in the high-fat diet groups. There is vacuolation in the granular layer of the dentate gyrus with the presence of a high number of glia cells (gliosis, G) in the molecular (ML) and polymorphic layer (PCL) of the dentate gyrus (D). 50X (H&E).

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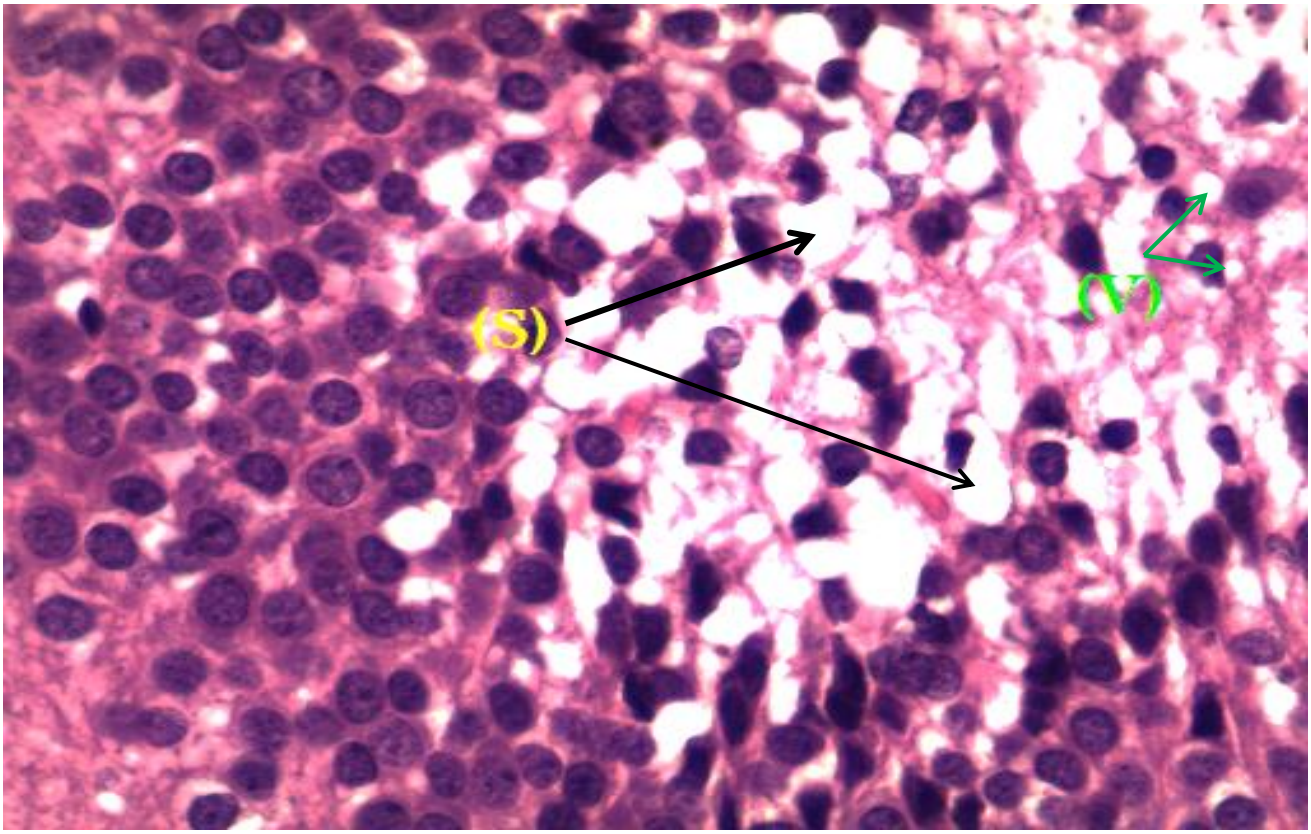

Figure 6. The brain tissue of a rat in high-fat group. The granular layer of the dentate gyrus shows the marked vacuolation of granular cells (V), which appear to be swelling (S) and vacuoles (V) in their cytoplasm and pyknotic nuclei. 200X (H&E).

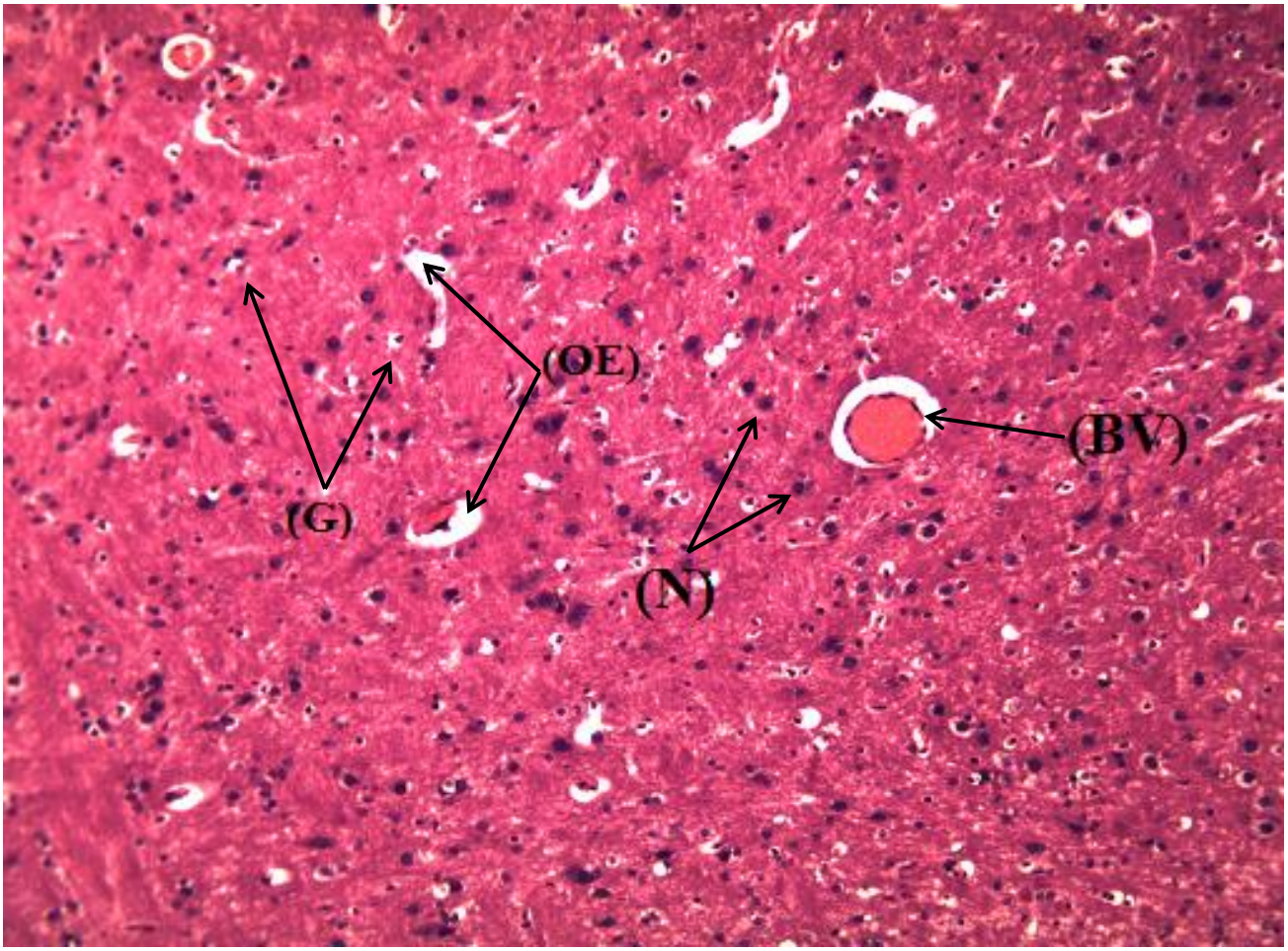

Figure 7. The brain tissue of a rat in high-fat diet group. The marked congestion in the blood vessels (BV) with marked cerebral edema (OE) in which there is dilation of Virchow-Robin space. Many neurological cells appeared shrunken and deeply stained (N). Infiltration of many glial cells (gliosis, G) and Astrocytes (Astrocytosis, A). 50X (H&E).

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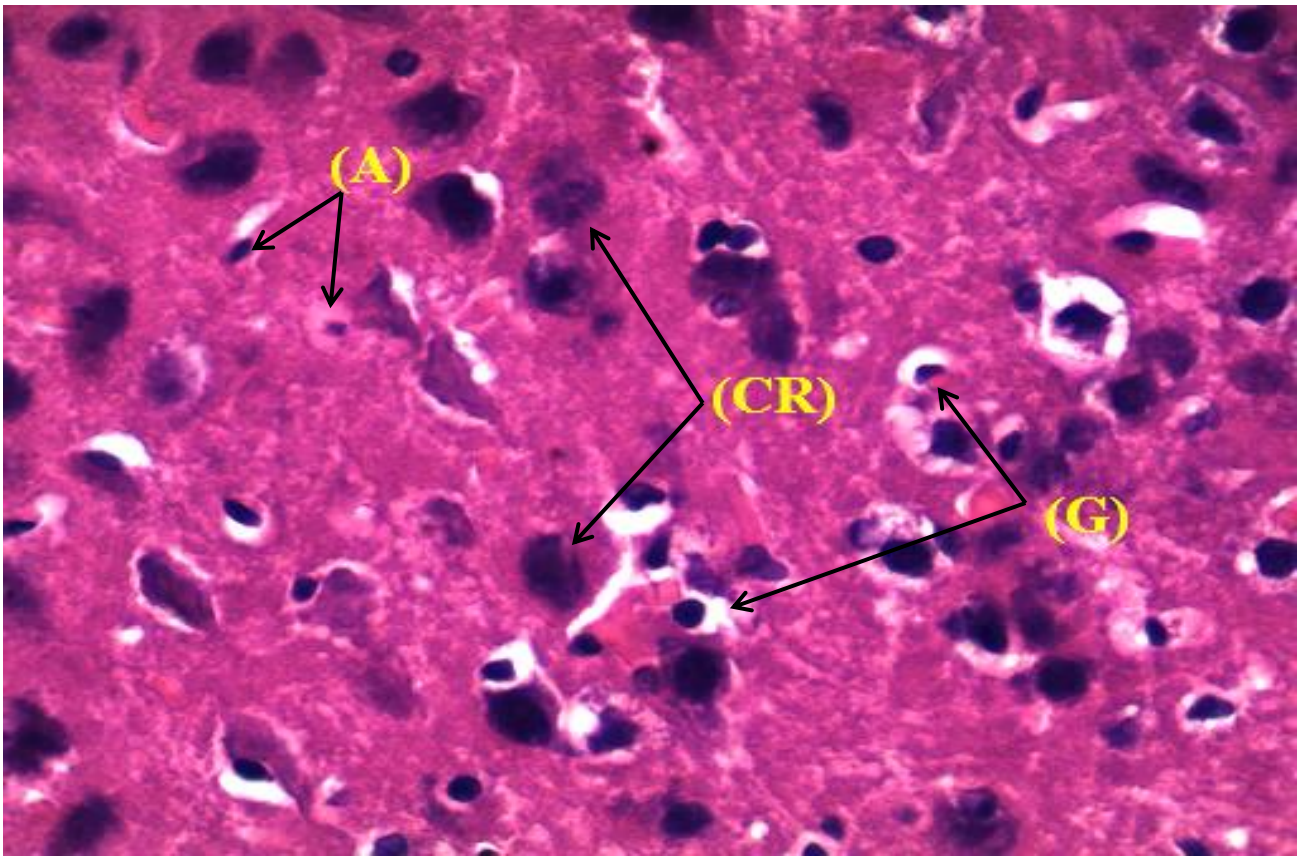

Figure 8. The brain tissue of a rat in the high-fat diet group. The neurological cell (neuron) shows marked central chromatolysis (CR) in which the neuron's body (perikaryon) shows spherical nuclei disappearance. There is gliosis (G) and Astrocytosis (A) in the brain tissue. 200X (H&E).

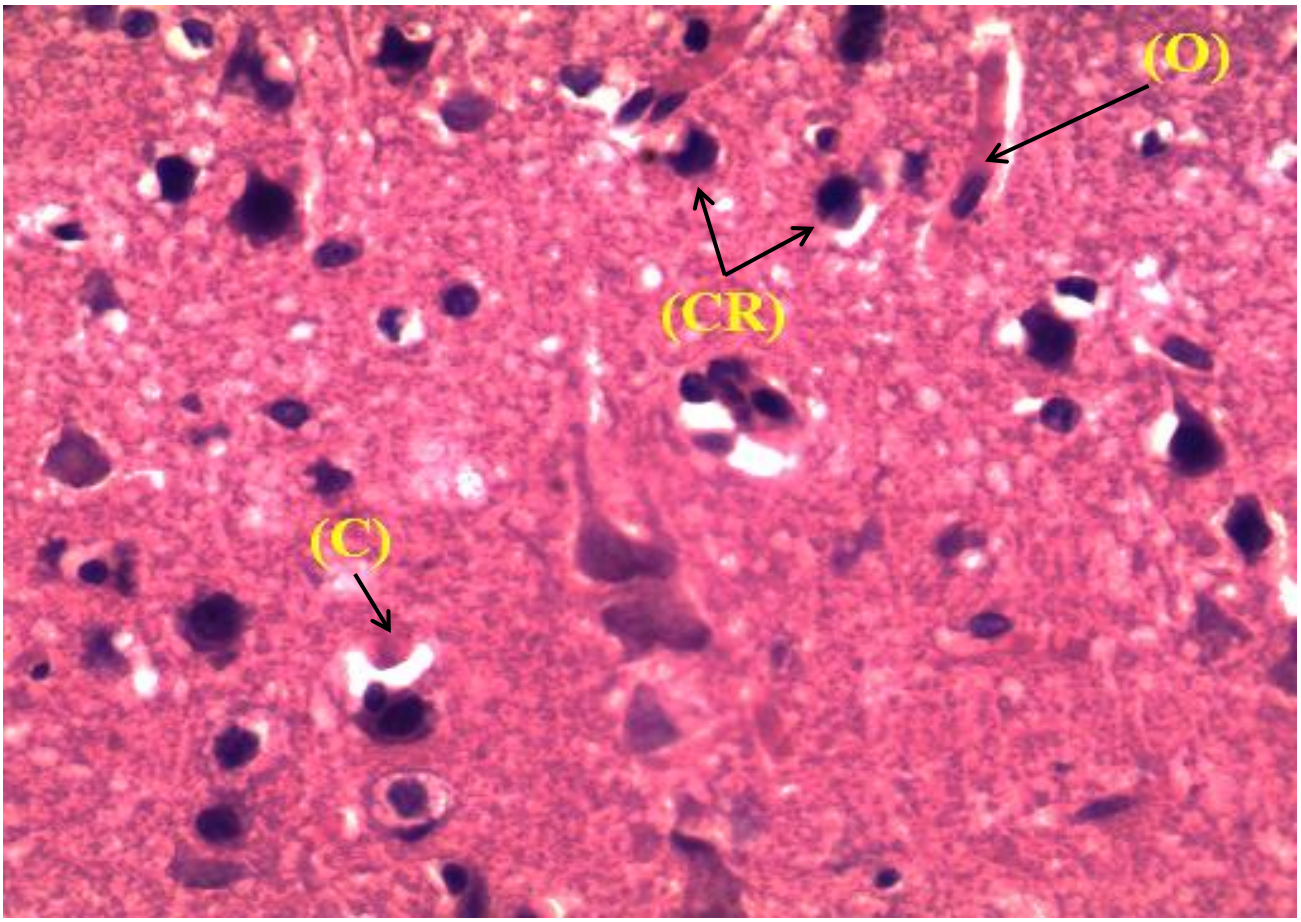

Figure 9. The brain tissue of a rat in high-fat diet group. There is gliosis and Astrocytosis with the presence of Oligodendrocytes (O). Marked central chromatolysis in the body of neurons (CR, spherical in shape and disappeared nucli). There is congestion (C) of fine capillaries in the brain tissue. 200X (H&E).

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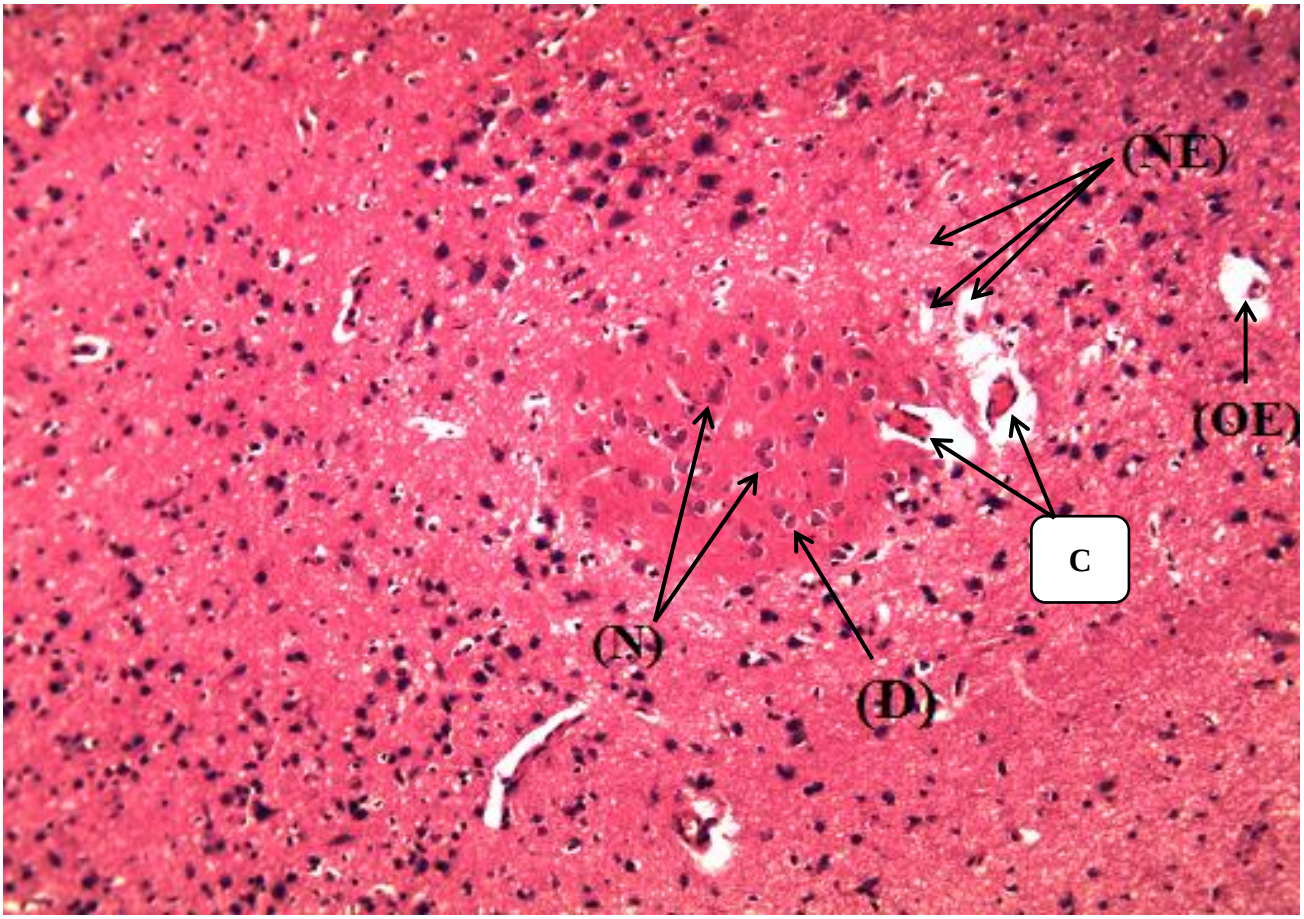

Figure 10. The brain tissue of a rat in high-fat diet group. Marked degenerative area thin the brain tissue (D) in which there is necrosis of neurons (N), the neuron showed pale in color and disappearance of nuclei with marked neurophagia in the brain tissue. Cerebral edema (OE) and congestion of blood vessels (C) are also seen. 50X (H&E).

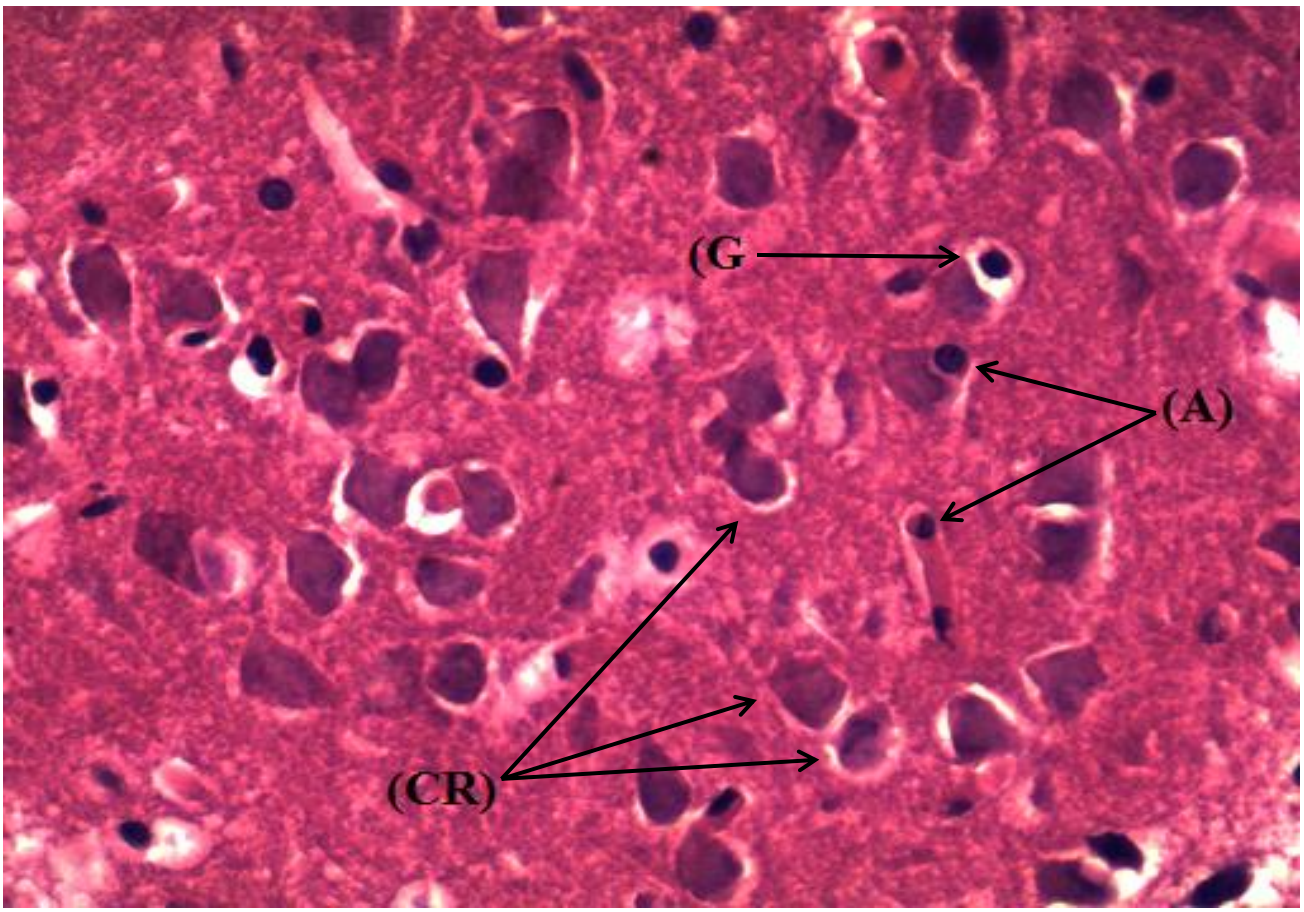

Figure 11. The brain tissue of a rat in the high-fat diet group. Severe central chromatolysis (CR)of perikaryon of neurons with gliosis(G) and Astrocytosis(A). 200X (H&E).

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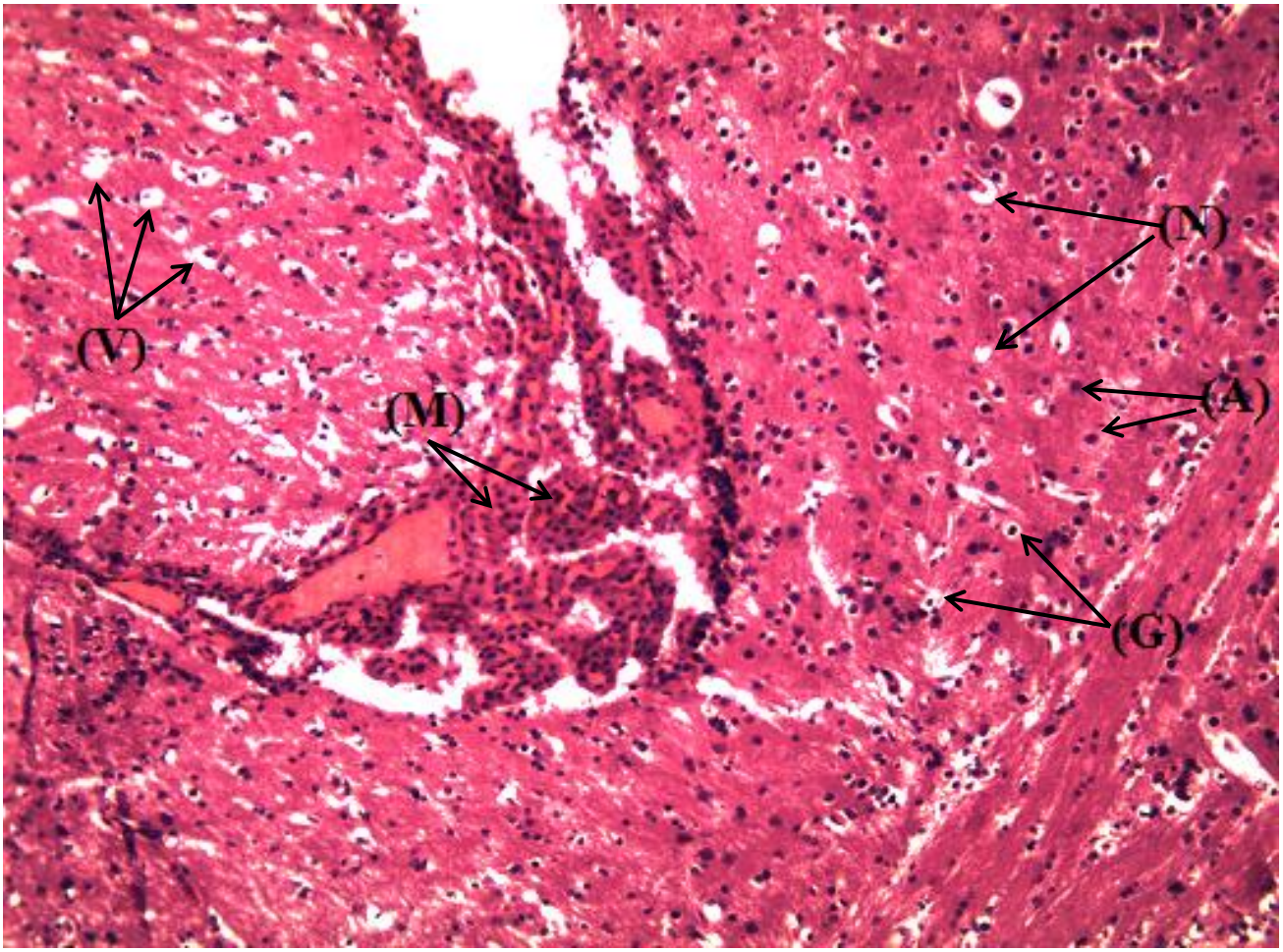

Figure 12. The brain tissue of a rat in high-fat diet group. Severe congestion and infiltration of inflammatory cells (M) in meninges (meningitis). Vacuolation (V) and neurophagia (N) in the brain tissue and marked gliosis (G) and astrocytosis (A) are noted in this section. 50X (H&E).

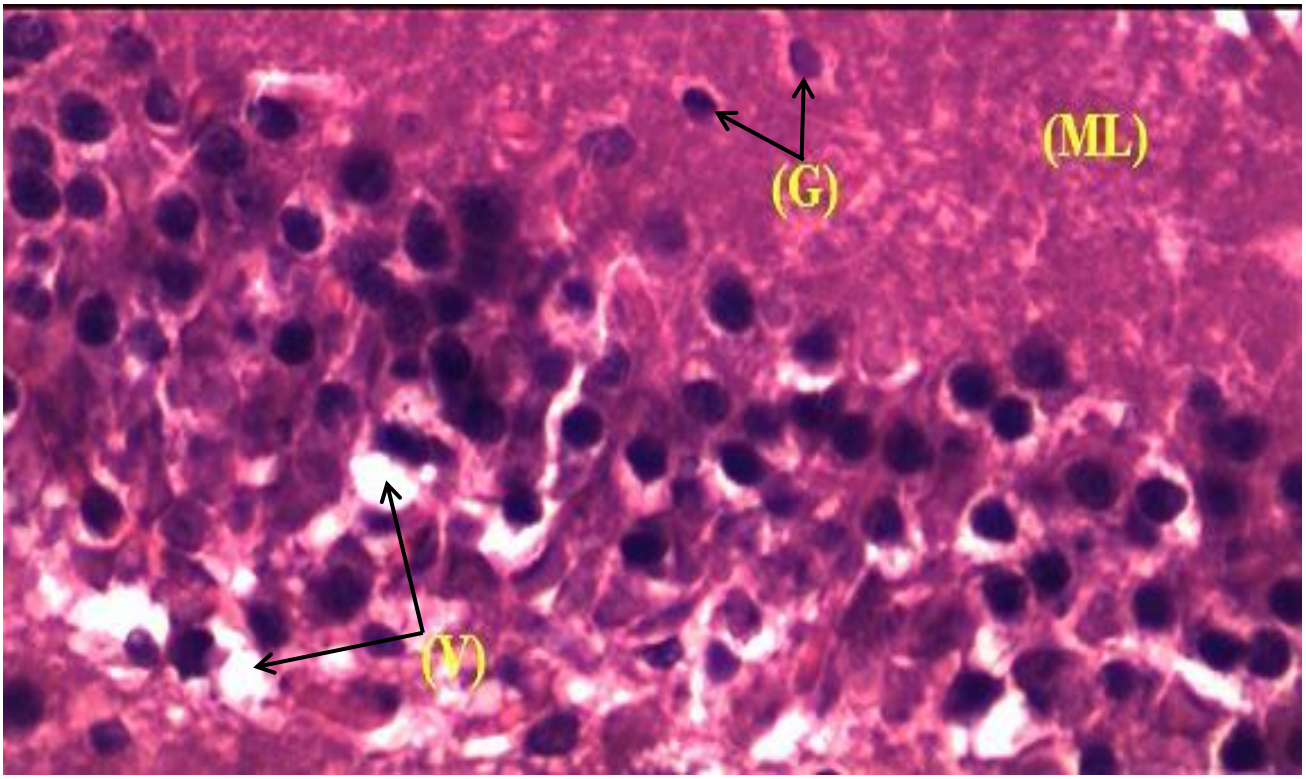

Figure 13. The brain tissue of a rat in the high-fat diet plus melatonin group. Few vacuolations in the granular cells (V) of the dentate gyrus, and most of these cells showed prominent nuclei. Few glia cells (G) in the molecular layer (ML) of the dentate gyrus. 200X (H&E).

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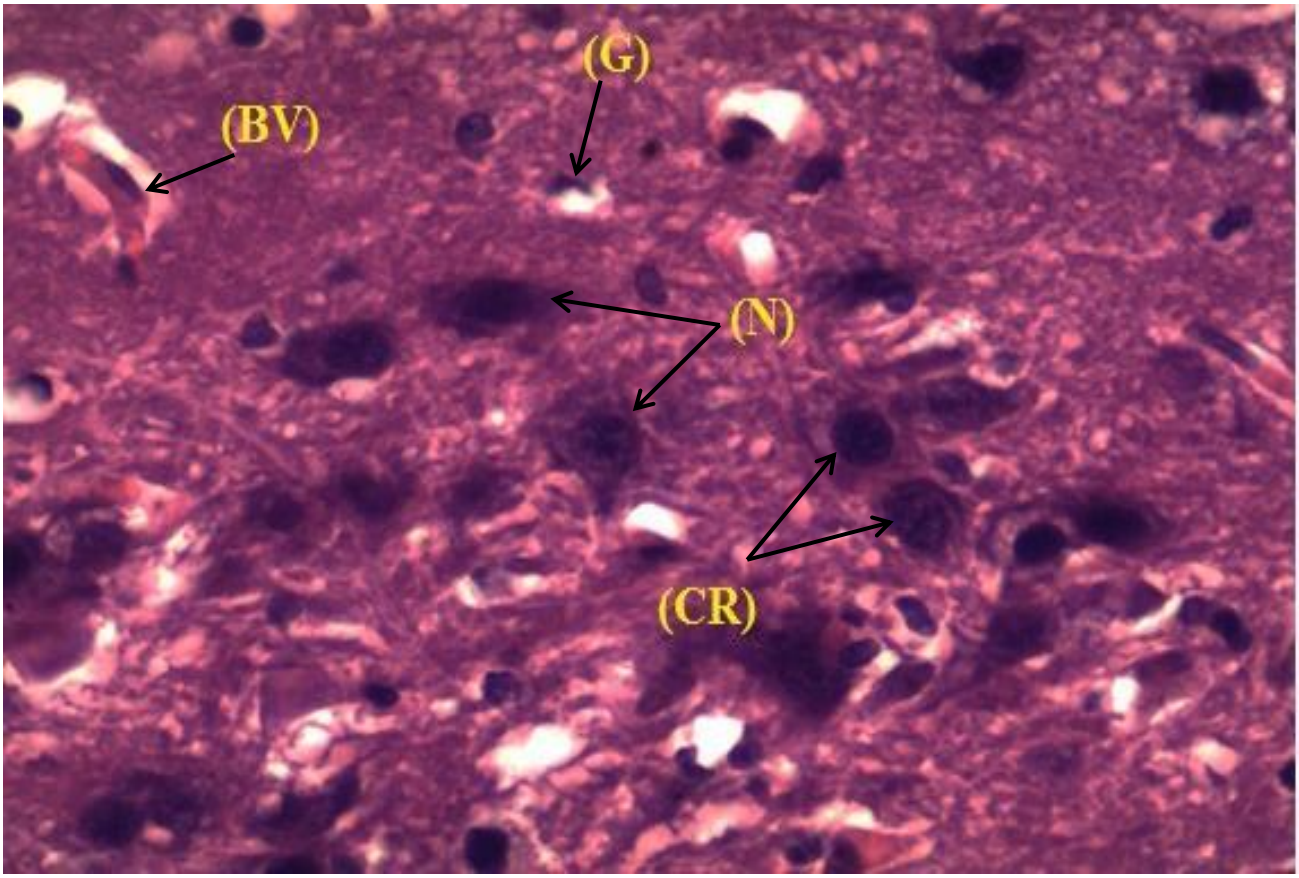

Figure 14. The brain tissue of a rat in the high-fat diet plus melatonin group. Most neuronal cells (neuron)(N) showed normal triangle perikaryon with central and prominent nuclei, but few of these cells suffer from central chromatolysis (CR). Normal blood capillaries (BV) and few glia cells (G). 200X (H&E).

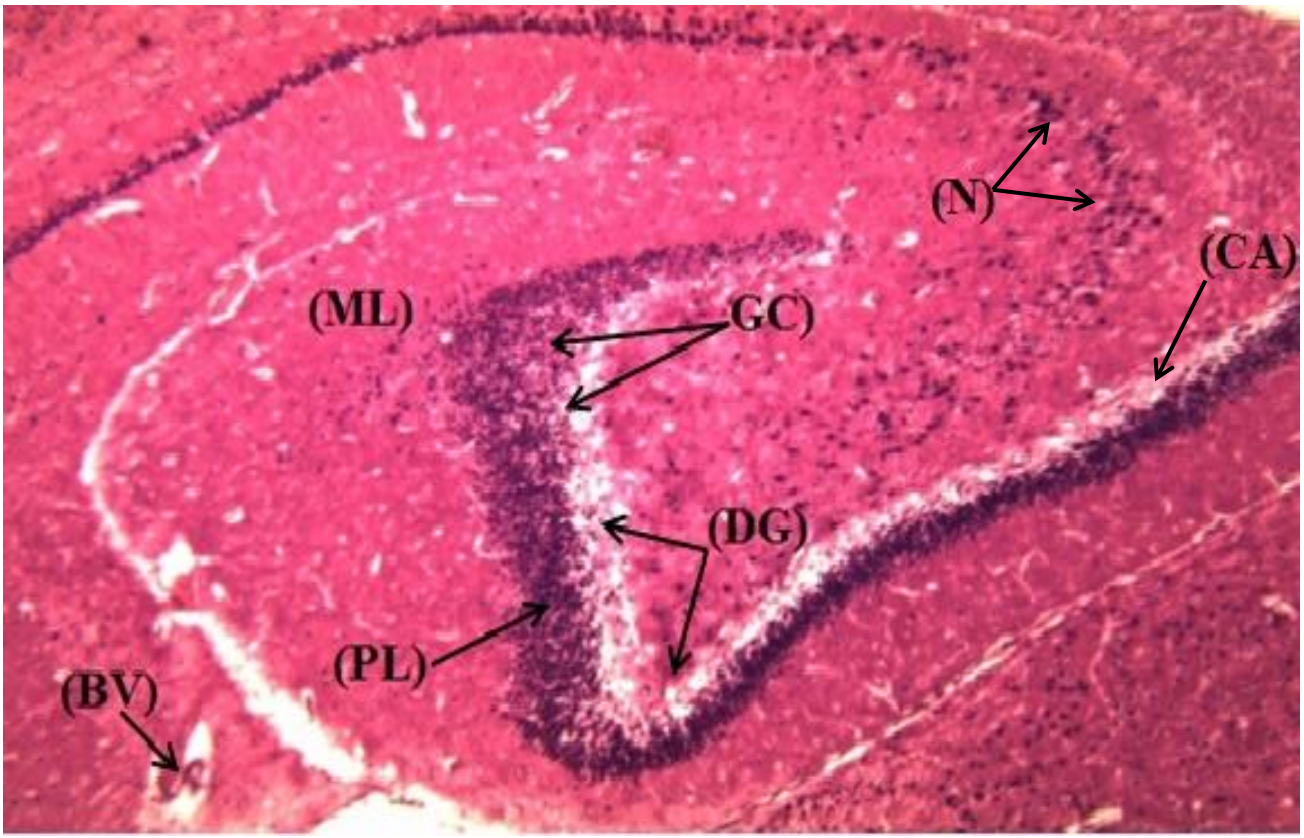

Figure 15. The brain tissue of a rat in high-fat diet plus melatonin group. The hippocampus showed nearly similar to the control. The first area of Cornu Ammonis (CA) is clear and thick, with a high number of pyramidal cells (PL) in the middle; there is a normal outer molecular layer (ML). But the inner polymorphic layer showed few congestions in the fine capillaries (BV). There are normal and high numbers with the normal shape of neurological cells (N) in the last area of cornu Ammonis. Thick dentate gyrus layer (DG) in which there is a high number and proliferation of granular cells with little vacuolation in it (GC). 20X (H&E).

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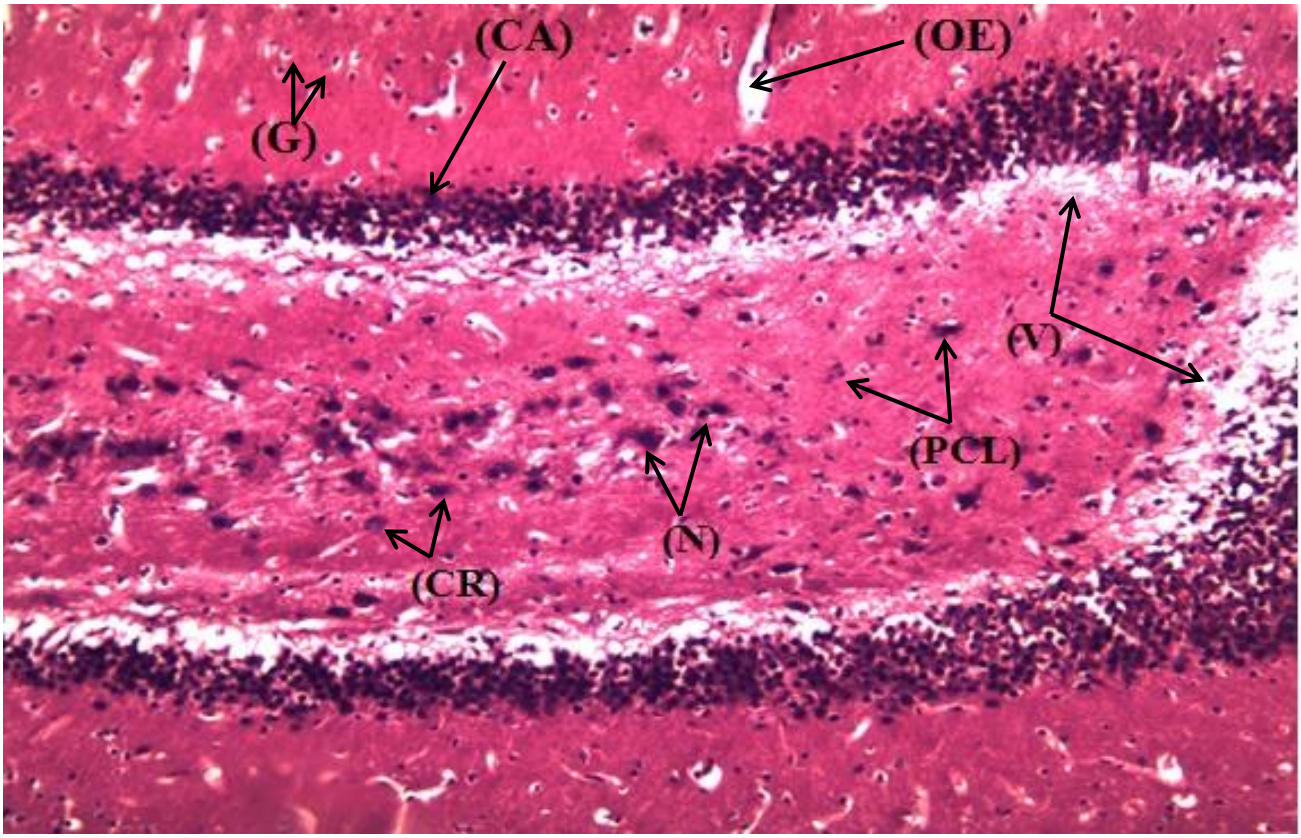

Figure 16. The brain tissue of a rat in the high-fat diet plus melatonin group. Most neurons showed normal with central nuclei in their perikaryon, but few neurons undergo chromatolysis (CR) in the last area of Cornu Ammonis (CA). Few vacuolation (V) at the granular layers of the dentate gyrus, mild perivascular edema (OE) in the molecular layer, and few glia cells (G) in the molecular layer and polymorphic layer (PCL) of the dentate gyrus. 50X (H&E).

DISCUSSION

The obtained findings were inconsistent with a recent study which revealed a dopamine decrease in male and female mice fed HFD, compared to the control group after a day and a week (Wallace et al., 2022). Not only significant effects on dopamine were reported, but fat rats were also shown to have lower levels of dopamine receptor D2 (DRD2) when fed a high-fat or high-sugar diet (Fritz et al., 2018). Moreover, another study indicated that 2 weeks of HFD consumption in adult Sprague Dawley rats induced disrupted dopamine networks, compared to the control group (Barnes et al., 2022). This result agrees with that of Han et al. (2021), showing that HFD-exposed mice had significantly higher midbrain DA and DRD2 protein levels compared to the control group. During the 8 weeks of HFD, obese rats showed 42% lower striatal DRD2 density and 30% lower total dopamine transporter (DAT) expression (Sharma and Fulton, 2013). These results agreed with a previous study that indicated lower striatal dopamine 2-receptor density, lower total dopamine transportal expression, and lower *in vitro* and *in vivo* dopamine transport function after an 8-week HDF exposure due to the positive correlation between circulating leptin and stress-induced dopamine release (Narayanaswami et al., 2013). The literature reviewed here suggests that leptin acts as a regulator of neuronal function and may provide an etiological mechanism for differences in dopamine neurotransmission in response to salient stimuli. When leptin is present, messages are sent to the brain's reward centers, where the dopamine system lowers the value of foods as a reward (Burghardt et al., 2012; Yeung and Tadi, 2020).

A high-fat, low-carbohydrate diet disrupts dopamine function linked to changes in brain neuroactivity due to dopamine dysregulation. Other behavioral-related neurochemical pathways may be impacted (Han et al., 2021). After 5 months of feed exposure, neurochemical studies showed that HFD-exposed mice had significantly greater DA and DRD2 protein levels in the midbrain, compared to the control group. They suggest that the effects of HFD on the C57BL/6J mice strain become apparent by the third month of dietary exposure (Han et al., 2021). Dopamine neurotransmission impairment has been linked to metabolic abnormalities produced by saturated fat (Serafine et al., 2016; Fordahl and Jones, 2017; Narayanaswami et al., 2013; Barnes et al., 2022).

However, it was indicated that dopamine levels were reduced in the melatonin groups, compared to the control group in a previous study (Lin et al., 2008). Previous studies have revealed that melatonin inhibits dopamine expression and serves as an antioxidant to prevent nigrostriatal neurodegeneration and alpha-syncline aggregation (Zisapel, 2001). Melatonin upregulation of the D2 receptor reduces the neuronal loss and downregulation of the dopamine transporter

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(Lin et al., 2008; Deng et al., 2015). Another investigation revealed that the group which received Acrylamide presented decreasing in neurotransmitters, such as dopamine. In contrast, dopamine was higher in the group that received Acrylamide and melatonin but still lower than in the control group as the melatonin protected the DNA damage and anti-inflammatory effect (Edres et al., 2021). Another study showed the IP therapy of 10 mg/kg body weight melatonin could lessen oxidative damage by boosting the activity of antioxidant enzymes, reducing lipid peroxidation and inflammation, and enhancing histopathological changes in the brain tissue of rats with pinealectomy (Bicer et al., 2022). Finally, melatonin may suppress neural nitric oxide synthase activity, leading to a decrease in NO and peroxynitrite formation (which play a critical role in the losses of dopaminergic neurons. This may inhibit the activity of tyrosine hydroxylase, a rate-limiting enzyme in dopamine synthesis (Teixeira et al., 2003; Ahmed et al., 2010; Pandi-Perumal et al., 2013).

A powerful pro-inflammatory cytokine called TNF is essential for starting and maintaining the inflammatory response. Elevated levels of TNF-α have been documented in several neurodegenerative disorders and insults with the potential to affect the Central Nervous System; for neurodegenerative diseases linked to neuroinflammation, controlling TNF-signaling may be advantageous (Frankola et al., 2011). The current experiment showed that the brain level of TNF-α cytokine was significantly higher in the HFD group, compared to other groups. These findings support the peripheral pro-inflammatory effects of inflammation of the used obesogenic diet. The current results of TNF-α, in parallel with many previous studies, revealed that the plasma biomarkers, TNF-α, and brain hippocampal significantly increased in HFD group rats, compared with the normal control rats (Xu et al., 2019; Ebrahim et al., 2021; Hsu et al., 2021). Further, the results of the study were similar to those of a previous study that examined HFD and inflammation. In rats who were given HFD for many weeks compared with those who were given a normal diet for the same period, TNF-alpha levels in the brain and different serum parameters were elevated (Spagnuolo et al., 2015).

The possible immunological mechanism that led to the elevation of TNFα has been explained in different studies. The Saturated fatty acids and lipopolysaccharides operate as agonists for the toll-like receptor 4 (TLR4), which it can bind to and activate. This causes nuclear factor kappa B (factor NF-B), leading to pro-inflammatory synthesis of cytokine, such as TNF-α (Rogero and Calder, 2018). Neuronal stress in response to HFD is associated with increased NF-kB signaling (Vykhovanets et al., 2011). Consuming an HFD raises oxidative stress and decreases mitochondrial activity in the brain (Tan and Norhaizan, 2019; Langley et al., 2020). Astrocytes and microglia produce substantial amounts of TNF in pathological settings; this de novo synthesis is a crucial part of the so-called neuroinflammatory response linked to several neurological disorders (Montgomery and Bowers, 2012). Although astrocytes and neurons can generate TNF-α, the microglia are believed to be the primary source of this cytokine during neuroinflammation (Chung and Benveniste, 1990; Welser-Alves and Milner, 2013). The cytokine interferon-gamma (IFN-α) is a strong inducer of TNF production in microglia (Mangano et al., 2012; Olmos and Lladó, 2014).

In the current study, melatonin administration restored the normal level of TNF-α in HFD+M. This agrees with previous investigations in which 10 mg/kg of melatonin was administrated for 20 days. The results indicated that administration of melatonin led to significant lowering levels of IL-1β, NF-κB, and TNF-α as the melatonin exerts anti-inflammatory influence by reducing TNF-α, interleukin-1β and iNOS, markers in diabetes mellitus (DM) induced animals (de Melo et al., 2020). However, in a recent study, it was reported that the administration rats with melatonin at the dosage of 500 µg/kg/day in diabetes mellitus DM rats for 6 weeks resulted in significant of the liver and adipose tissues TNF and NF-κB lower than in the control animals (Yapislar et al., 2022). Moreover, it has been demonstrated that melatonin inhibits the synthesis of pro-inflammatory cytokines, such as TNF-α. Rats had significantly lower levels of the transcription factor NF-$_{k}$B, which is crucial for innate immunity and mediates the production of the pro-inflammatory cytokines TNF-α (Somade et al., 2019; Brazão et al., 2022). On the other hand, another study showed the effect of melatonin on ischemic brain injury in the apoptotic response and inflammation in aged rats. It was found that the animals given melatonin starting 24 hours before surgery and continuing for the first 7 days after an ischemic stroke led to oxidative stress, blood-brain barrier dysfunction, post-ischemic inflammation, and microvascular injury. Moreover, there was a significant decline in tumor TNF-α and interleukin-1 beta (IL-1 beta) levels (Rancan et al., 2018). Prior research has shown that melatonin can prevent the transcriptional activation of IL1 and TNF-α by blocking NF-κB binding to DNA (Chuang et al., 1996; Li et al., 2005; Farid et al., 2022). In addition, melatonin therapy attenuates the inflammatory signals triggered by insulin resistance/hyperinsulinemia, reducing adipose/hepatic inflammation (Obayemi et al., 2021).

The recorded findings agreed with a previous study that revealed that a diet rich in fat drastically lowered spatial working memory performance in the Y-maze test (Ajayi et al., 2021). Moreover, feeding rats with a diet rich in fat for two months impaired their learning ability, and rats took longer and traveled a longer distance compared with the control in the Morris Water Maze task (Sepehri et al., 2019). There is still much mystery about how a high-fat, low-carbohydrate diet can impair mental acuity. Multiple studies have shown HFD to increase oxidative stress and free radical generation, leading to lipid peroxidation and changes in the blood-brain barrier's structural components (Reiter et al., 2014; Alzoubi et al., 2017; Alzoubi et al., 2018). Moreover, Abdulwahid (2019) stated that HFD impaired hippocampal neural function by impairment of inhibitory neurotransmitters. Furthermore, the activation of astrocytes might be a remarkable indication of inflammation in the hippocampus (Abdulwahid, 2019). Neuronal damage markers, such as serotonin, dopamine, and

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glutamate were significantly altered in the brains of HFD rats, which may explain the resulting apoptosis of hippocampus cells and cognitive impairment (Labban et al., 2020; Parkington et al., 2020). it was suggested that melatonin improved spatial learning and memory by reducing isoflurane-induced endoplasmic reticulum stress and neuroapoptosis in the hippocampus and serum levels of neuroinflammatory markers (Fang et al., 2022). A decrease in the inflammatory response in pro-inflammatory cytokines, such as interleukin-1 (IL-1), and tumor necrosis factor Alfa (TNF-α), and an increase in anti-inflammatory cytokines like IL-4 have been observed in an animal study where exogenous melatonin was administered before acute conditions (Carrasco et al., 2013). In addition, melatonin reduces the formation of high amounts of prostanoids and leukotrienes, and other mediators of the inflammatory process, such as chemokines and adhesion molecules, by inhibiting the expression of cyclooxygenase and inducible nitric oxide synthase (iNOS, Liu et al., 2017). To measure the anxiety-like behavior in experimental animals, Elevated plus maze (EPM) was used. The current EPM findings agreed with previous studies, which reported that an HFD impairs assessed anxiety-like as well as working memory behaviors by decreased open arm time in the EPM, and increased movement and rest episodes and decreased rearing in the open field test (Hu et al., 2017; Holl et al., 2018; Deal et al., 2020). Previous studies on the cognitive deficits and anxiety-like behavior in rodents fed an HFD indicated that indoleamine 2,3-dioxygenase (IDO) activity and pro-inflammatory cytokines were involved in the proposed mechanism (André et al., 2014; Parkington et al., 2019). On the contrary, Kaczmarczyk et al. (2013) reported that HFD rapidly impacts dopamine metabolism in the brain, appearing to trigger anxiety-like behaviors and learning/memory impairments. In the reduction of the ratio of reduced glutathione GSH: oxidized glutathione GSSG, by elevation of cytosolic reactive oxygen species, anxiety-like behaviors in mice can be observed (Llorente-Folch et al., 2013). Finally, in rat research, it has been demonstrated that amphetamine withdrawal causes depression and anxiety-like behavior associated with DA dysregulation. In the hippocampus and amygdala, the dopamine D1 and D2 receptors are associated with anxiety-related behaviors (Svingos et al., 2001; Lee et al., 2018).

In a sporadic rat model of Alzheimer's, melatonin administration during the disease's active stage of progression decreased amyloid deposition in the hippocampus (β1-42 and β1-40) and frontal cortex (β1-42), decreased degenerative changes in the hippocampus, prevented mitochondrial dysfunction, and postponed anxiety and cognitive decline (Rudnitskaya et al., 2015). Prolonged melatonin use prevents neurodegeneration in rat's hippocampus by reducing hyperphosphorylation and Aβ mediated memory impairments following intracerebroventricular Aβ1-42-injection (Ali and Kim, 2015). After intracerebroventricular injections of soluble A1-42, it has also been found that melatonin enhances spatial memory, decreases astrogliosis in the rat hippocampus, and decreases synaptic dysfunction (Zhang et al., 2016). In a sporadic rat model of Alzheimer's disease, it has been discovered that long-term oral melatonin administration and maintains the neuronal and glial structure increases hippocampus synaptic development (Stefanova et al., 2015). Previous studies have revealed that feeding heavily on fat and carbohydrates can increase blood-brain barrier permeability and impair cognition (Davidson et al., 2013). These results confirm that rats administered HFD have lower hippocampus plasticity. Additionally, hippocampus volume shrinks, and the hippocampus volume will inevitably decline due to neuronal death (Cherbuin et al., 2015).

An electron microscopy analysis revealed that rats fed an HFD had degenerative neurons, swallowed mitochondria, expanded endoplasmic reticulum cisterns, and increased lysosomes and vacuoles (Alkan et al., 2021). Similar to previous studies, inflammation caused by fatness leads to various disorders and affects the nervous system. In the CNS, mild cognitive impairment can be attributed to obesity-induced altered hippocampal structure and function (D O'Brien et al., 2017).

Rats on the HFD displayed decreased mRNA expression of the blood-brain barrier capillary system's tight junction proteins, Claudin-5 and Claudin-12. The claudins, among the tight junction proteins, are thought to be the main proteins that create the tight junction features of the endothelial cells and are significant in permeability restriction (Wolburg and Lippoldt, 2002). The HFD was also connected to increased sodium fluorescein (NaFl) permeability from the vasculature to the hippocampus region, which is consistent with the lower production of these proteins (Kanoski et al., 2010). Accordingly, such a diet can alter the brain's internal environment by disrupting the function of the blood-CSF and blood-brain barriers. In conjunction with the hippocampus' apparent vulnerability to increased NaFl permeability, behavioral findings imply that this brain area is more susceptible to the neurotoxic effects of saturated fats and refined carbohydrates on blood brain barrier permeability and cognitive function (Hargrave, 2014; Clasen et al., 2020). By making contact with the endothelial basal lamina, astrocytes control leptin's capacity to penetrate the blood-brain barriers (Hsuchou et al., 2009). According to the previous study, reactive gliosis alters the production and function of these connexins proteins in astrocytes, particularly via opening the connexins hemichannel (Giaume et al., 2021). Certain circumstances, such as reduced pH, oxidative stress, as well as inflammation brought on by injuries, may cause the hemichannel to open (Turovsky et al., 2020). In addition, gliotic astrocytes may directly contribute to tissue injury through local proinflammatory signals. Similar to microglial activation, astrogliosis is triggered by an HFD in mice. This gliosis can be produced by several factors, including the release of nitric oxide (NO, Gzielo et al., 2017). Fat and cholesterol-rich diets are a known risk factor for cognitive decline due to their effects on hippocampus-dependent

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memory, blood-brain barrier dysfunction, and hippocampal neurogenesis (López-Taboada et al., 2020). It is worth mentioning that the changes occurring in the brain tissue are due to oxidative stress, changes in the mitochondria, and inflammation evident in the microscopic examination through the increase of cells that show inflammation. The majority of the cited research revealed that the effects of insulin on the tissues lead to alterations, which was supported by biochemical investigation through the brain tissue by TNF examination. A previous study revealed Lipid peroxidation reduced after the melatonin infusion, most notably in the brain. An increase in antioxidants, such as GSH-Px activity was also observed after melatonin treatment. This means that melatonin increases the levels of the antioxidative enzyme GSH-Px and removes free radicals (Baydaş et al., 2001). Melatonin inhibited lipid peroxidation in the mitochondria and the cytoplasm in a different experiment that looked at its capacity to prevent oxidative damage in brain tissue and decrease antioxidative enzyme activity in brain tissue (Shen et al., 2002). Another recent study by Bicer et al. (2022) about the neuroprotective of melatonin gains brain damage so melatonin increased the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) and improved brain total antioxidant status (TAS) and suppressed lipid peroxidation, inflammatory pathways, and apoptosis. Treatment with melatonin considerably improved the histological features in the hippocampus, cortex, and cerebellar region, which included vacuolation, inflammatory cells, and pyknotic cells (Sarena et al., 2022). Moreover, melatonin applied before radiotherapy protects against early-period radiotherapy-induced brain damage (Aras et al., 2021). Thus, results indicate that melatonin therapy could reduce reactive gliosis. Due to its great efficacy, low toxicity, and ability to cross the blood-brain barrier, it had a neuroprotective impact that reduced cell death and reactive astrogliosis (Alonso-Alconada et al., 2012). The result agreed with a recent study that melatonin protects memory, oxidative stress, and inflammation when dopamine decreases (Amin et al., 2021). Some suggest that melatonin could inhibit the intrinsic pathway of apoptosis to prevent neurodegenerative diseases (de Lima et al., 2005; Tuzcu and Baydas, 2006; Ferreira et al., 2010). This confirms the role of melatonin in pathophysiological mechanisms, such as edema in the central nervous system and peripheral organs (Xu et al., 2017). In a similar study on histopathological examination, melatonin significantly reduced the rates of necrosis, neuronal degeneration, and edema (Erol et al., 2004). The ability of melatonin to stop neuroinflammation caused by a diet rich in fat and heavy sugar in an HFD-T2DM rat model by restoring pancreatic function, reducing adipose tissue mass, and easing dyslipidemia. This will decrease systemic inflammation, eventually stopping neuroinflammation by reducing oxidative stress in the brain and stopping the expression of iNOS (Maher et al., 2020). These results conclude that melatonin's antioxidant activities improve the brain tissues, the neuroprotective ability of melatonin, and the daily administration of 10 mg/kg /body weight of melatonin. These results agreed with a previous study (Favrais et al., 2021), which indicated that the IP injection of melatonin (5 mg/kg/daily) had promising neuroprotective effects due to its antioxidant, anti-inflammatory, and anti-apoptotic properties.

CONCLUSION

Feeding animals with high-fat diets impaired brain neurotransmitters and induced pro-inflammatory changes, as well as affected learning ability and memory by changing the structure of neural tissue. Injection of melatonin at a dose of 10 mg/kg/daily can repair the harmful effect of HFD and restores the normal histological structure of the brain, and biochemistry function, such as dopamine. Future studies are suggested to investigate the effect of different doses of melatonin and its injection time for assessing memory and cognition using Open field test, forced swim test, and Radial arm maze.

DECLARATIONS

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Authors' contributions

Dr. Ammar A. Abdulwahid conceptualized the idea and developed an experiment to conduct the laboratory work. Ahmed Raheem Rayshan, Ammar Ahmed Abdulwahid and Alyaa Abdulhussein Alsaedi analyzed data and contributed to the drafting, editing, and production of the final draft. All authors checked and approved the final version of the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

Availability of data and materials

The data presented in this study are available on request from the corresponding author.

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Treatment Effects of Chitosan Nanoencapsulated Bromelain against Gastrointestinal Nematodes and Coccidia in Goats of Kenya

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ABSTRACT

The management of gastrointestinal nematodes (GIN) and coccidiosis of livestock relies on the use of commercial anthelmintic; however, the excessive and frequent usage of these drugs has led to the substantial and dramatic development of anthelmintic and anticoccidial resistance. The present study aimed to evaluate the anthelmintic and anticoccidial efficacy of chitosan nanoencapsulated bromelain (CNB) against a wide spectrum of GIN and coccidia in goats. Additionally, the study assessed the safety of CNB in the goats. Bromelain was extracted from the pineapple peels and then encapsulated using chitosan. A total of 20 healthy male goats naturally infected with GIN and coccidia were used. The goats were separated into four treatment groups, with five goats per each. The CNB was orally administered at dosages of 270 and 90 mg/Kg, once daily for 60 days. Fecal egg counts (FEC), fecal oocyst counts (FOC), packed cell volume (PCV), aspartate aminotransferases (AST), alanine aminotransferases (ALT), urea, and creatinine were determined weekly. The goats were monitored for clinical signs daily, and their body weight was recorded weekly. The findings revealed that FEC reduction rates in the group that received 270 mg/Kg CNB and the group that received Albendazole were 73.41% and 79.54% at day 7 post-treatment. Also, the reduction of FOC in the group receiving 270 mg/Kg CNB at day 7 (84.12%) did not show a significant difference with Diclazuril (82.12%). The FEC and FOC were zero (reduction of FEC and FOC was 100%) at 28 days of treatment in goats treated with 270 mg/Kg CNB. During the monitoring period, no mortalities and no clinical signs were observed in the treated goats. The PCV, AST, ALT, creatinine, and urea levels for the goats in all groups were within normal limits. No pathological lesions were observed in the goat's organs. In conclusion, the results demonstrated that repeated (60 days) dosages of 270 mg/Kg had anthelmintic and anticoccidial effects and were safe for goats. The study recommends further investigation in a field setting involving more animals. This would allow the development of a novel product for managing helminthiasis and coccidiosis in ruminants.

Keywords: Anthelmintic, Anticoccidial efficacy, Bromelain, Chitosan, Encapsulation, Goat

INTRODUCTION

Endoparasites such as gastrointestinal nematodes (GIN) and coccidia are a serious health concern in small ruminants across the world (Jansen et al., 2020; Wasso et al., 2020; Wondimu and Bayu, 2022). In many tropical countries, the main GIN affecting small ruminants is *Hemonchus contortus*, *Oesophagostomum* spp., *Trichostrongylus* spp., *Ostertagia* spp., *Nematodirus* spp., and *Cooperia* spp. (Maichomo et al., 2004; Waruru et al., 2005). These parasites cause a decline in milk and meat production resulting in severe economic losses in the livestock industry (Waruru et al., 2005; Ibrahim et al., 2014; Mat Yusof and Md Isa, 2016). On the other hand, coccidiosis is caused by protozoan parasites of the genus *Eimeria* with the main one being *Eimeria ninakohlyakimovae, E. hirci, E. caprina, E. christenseni, E. jolchijevi, E. apsheronica,* and *E. arloingi* (Etsay et al., 2020). These parasites contribute to enteric disease, particularly in young or stressed livestock leading to low production and high mortality (Faizal and Rajapakse, 2001; Mohamaden et al., 2018; Etsay et al., 2020). In most of the ruminants' production systems, GIN and *Coccidia* manifest as mixed or singles infections which compound the morbidity and mortality cases (Faizal and Rajapakse, 2001; Jansen et al., 2020; Bawm and Htun, 2021). In Kenya, the prevalence of gastrointestinal tract parasites in small ruminants is high, with the prevalence of up to 82% and 85% for GIN and coccidiosis, respectively (Maingi and Munyua, 1994; Waruru et al., 2005; Kanyari et al., 2009).

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The management of GIN and coccidiosis of livestock relies heavily on the use of commercial anthelmintic and anticoccidial drugs (Kaplan, 2004; Noack et al., 2019). However, the excessive and frequent usage of these drugs has led to the substantial and dramatic development of anthelmintic and anticoccidial resistance to GIN and coccidia, principally in cattle, sheep, and goats (Hema et al., 2015; Mickiewicz et al., 2021; Potârniche et al., 2021). Regarding the growing threat to animal health and production posed by drug resistance, innovative medications, including those based on plant extracts need to be considered and developed as potentially sustainable alternatives (Sadr et al., 2022).

One of the potential anthelmintic and anticoccidial plants is pineapple (*Ananas comosus*), containing cysteine proteinases enzyme (Misran et al., 2019). The pineapple enzyme, bromelain, has been demonstrated to have anthelmintic and anticoccidial activities *in vitro* and *in vivo* levels (Githiori, 2004; Hunduza et al., 2020; Wasso et al., 2020; Daiba et al., 2022). The recent *in vitro* and *in vivo* studies by Wasso et al. (2020) and Daiba et al. (2022) called for the investigation of the efficacy of nanoencapsulated bromelain at enhanced and repeated doses. The study by Wasso et al. (2020) reported that nano-encapsulated bromelain caused an efficacy of up to 68.8% at 30 mg/Kg for 14 days of treatment. The aim of the current study was to use higher doses of nano-encapsulated bromelain to investigate the *in vivo* efficacy and toxicity of encapsulated bromelain in chitosan against GIN and coccidia in goats.

MATERIALS AND METHODS

Ethical approval

The approval for the goats' experiments was obtained from the Animal Ethics Committee of the University of Nairobi, Kenya (REF: FVM BAUEC/2020/339). The study followed the design, animal husbandry practices, and protocols approved by the Committee.

Experimental site

This controlled laboratory study was carried out between July 2021 and January 2022, at the animal facility located at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja Campus, located just outside Kenya's capital city of Nairobi. The University is located at latitude 1°05 S and longitude 37°00 E, and it lies at an altitude of 1525 m above sea level with rainfall bimodal and ranges from 500 mm - 1300 mm while the average temperature is 19.5°C (Menge et al., 2014).

Extraction and encapsulation of bromelain in chitosan

The University Campus falls within the agricultural zone, where commercial pineapple cultivation forms a substantial part of agricultural activities. Pineapples were readily available at the local market. Bromelain was extracted from the peels of pineapples (*Ananas comosus*) sold at a local market in Juja sub-county, Kiambu County, Kenya. The bromelain was extracted using the procedure described previously by Hunduza et al. (2020). Fresh ripe pineapples were ground in a blender in sodium acetate buffer (pH = 7.4). The resultant crude extract was precipitated by adding 40% ammonium sulfate. After 24h of incubation at 4°C, extracted bromelain was purified using a dialysis membrane (size of 12 kDa). Bromelain in chitosan (Sigma Aldrich, USA) was encapsulated by the ionic gelation method (Hunduza et al., 2020). The pellet obtained after encapsulation was dried by placing it in the freeze-dryer (MRC, Model FDL-10N-50-BA, Israel) after frozen at -60°C. The Fourier transform infrared spectrophotometer analysis (FTIR) was used to confirm the success of the encapsulation of bromelain in chitosan nanoparticles (Hunduza et al., (2020).

Experimental animals

Twenty small East African male goats, which were naturally infected with GIN and coccidia, were purchased from farmers (before purchase, fecal samples were collected to diagnose positive animals for both GIN and coccidia infection) in Makima Ward in Embu County in Northeastern Kenya. Molecular identification by PCR showed that animals were infected by a wide number of nematodes (*Haemonchus contortus*, *Trichostrongylus vitrinus*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Nematodirus filicollis*, *Oesophagostomum* spp., and *Ostertagia ostertagi*). The average age of the goats was 15 months old, and they weighed between 22-31 Kg. Acclimatization to the diet was done for 2 weeks, and animals were tagged with ear tags for easy identification before the start of the experiment. The goats were group-housed at the JKUAT animal facility. The animals were kept in pens of 3 m x 3 m size and were fed with 1.5 Kg of wheat hay thrice per day, 1 Kg/animal of feed concentrate made up of beet liquid molasses, maize germ, and soybean meal (Aroma Feed Suppliers®, Kenya). The animals had access to essential minerals lick blocks (Aroma Feed Suppliers®, Kenya) and fresh drinking water *ad libitum*.

Treatment groups and sampling

Fresh fecal samples (10 grams) were collected using sterile gloves from the rectum of goats. These were analyzed to determine the number of eggs per gram (EPG) and coccidia oocysts per gram (OPG) of feces using the McMaster method (Cringoli et al., 2004). The animals formed self-control samples taken at the start of the study. All the animals

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used in the experiments had an EPG and OPG of more than 2,000 and 10,000, respectively. The goats were divided into four treatment groups of five goats. Groups 1 and 2 received 90 mg/Kg and 270 mg/Kg of CNB, respectively, while group 3 was considered as the positive control where goats were administered orally with 7.5 mg/Kg of Albendazole (Sigma Aldrich, USA) and 5 mg/kg of Diclazuril (Sigma Aldrich, USA), and group 4 was served as negative control (untreated goats). The above dosages were chosen based on the results of the bromelain toxicity and efficacy tests obtained in the previous studies (Wasso et al., 2020; Daiba et al., 2022). The treatment was done orally every morning for 60 days. The animals were monitored for 90 days after the last day of drug administration.

***In vivo* assessment of anticoccidial efficacy**

Fecal samples were collected from the rectum of goats every 7 days after the start of drug administration up to the end of the monitoring period (150 days). The fecal samples were analyzed using a modified McMaster technique to determine the EPG and OPG (Coles et al., 1992; Odden et al., 2018). Fecal egg count reduction (FECR) and fecal oocyst count reduction (FOCR) percentages were evaluated as previously described by Coles et al. (1992) and Odden et al. (2018).

Assessment of toxicity effect

Following dosing, the animals were observed for acute toxicity during the first 30 minutes and then every 6 hours during the first 24 hours. Thereafter, the animals were monitored for any changes in general behavior, clinical and other physiological activities (OECD, 2002; Parasuraman, 2011). Rectal temperature of goats was measured daily, each morning (8 a.m. to 9:30 a.m.) coinciding with the time of drug administration) using a digital thermometer (Kruuse Digital Thermometer; Jorgen Kruuse). The body weight of animals was recorded weekly using a 100 Kg spring balance scale (Salter Model, Capital Scales - Pretoria, South Africa). Two mL of blood was sampled weekly from each animal from the jugular vein into a blood collection tube with 4 mL EDTA. The PCV was determined using the micro-hematocrit method (Shamaki et al., 2017). The plasma samples obtained were used to determine the levels of Aspartate aminotransferases (AST), Alanine aminotransferases (ALT), urea, and creatinine using standard diagnostic test kits on an Automated Clinical Biochemistry analyzer (Reflotron Plus System®, model: Cobas 4800 Detection Analyzer; India, Emma et al., 2020; Wasso et al., 2020).

Following the last day of drug administration, the goats (2 goats per group on day 60 and the rest on day 150) were sacrificed using 0.25 mL/Kg of Pentobarbital sodium (Leary et al., 2020) and gross pathology conducted according to the procedure described previously by King et al. (2013). Sections of liver, kidney, spleen, and heart were collected and preserved in 10% buffered formalin for 24 hours before being processed for histopathology (Rousselle et al., 2019).

Statistical analysis

Data were entered into Microsoft office excel (Microsoft® Office 365™, Microsoft, USA) and analyzed using Graph Pad Prism 8.4.3 (San Diego, CA, USA) for data analysis. Descriptive statistics (means and standard deviations) were determined. The FECR, FOCR, weight, temperature, PCV, and biochemical parameters of different groups were compared with those of non-treated goats using Students t-test ($p < 0.05$).

RESULTS

Efficacy assessment of chitosan nanoencapsulated bromelain

The results showed a relatively high reduction in EPG count mean in all treated goats (Figure 1). On day 7 of treatment, 73.41% (580 EPG), 62.61% (780 EPG), and 79.45% (530 EPG) of reduction of FEC were recorded for goats treated with 270 mg/Kg, 90 mg/Kg of CNB and Albendazole, respectively. Contrariwise, the FEC increased by 107.42% in the untreated group (negative control). The reduction in FEC count reached 100% between day 28 to 77 post-first day of treatment for goats treated with 270 mg/Kg of CNB. During the first month of treatment, there was no significant difference ($p > 0.05$) in fecal egg counts in goats treated with 270 mg/Kg of CNB and the group treated with 7.5 mg/Kg of Albendazole. In the negative control group, the FEC increased by 127.18% on day 28.

Efficacy assessment of chitosan nanoencapsulated bromelain on fecal oocyst count reduction

At the start of the study, the mean OPG in goats in all the groups was similar. Following treatment, the oocyst count reduced gradually between days 7 and 35 of treatment (Figure 2). The reduction percentage in oocyst counts on day 7 post-treatment was higher ($p > 0.05$) for the goats treated with 270 mg/Kg CNB (84.12%, 2,660 OPG) and Diclazuril (82.12%, 3,180 OPG) than those treated with 90 mg/Kg CNB (44.83%, 7,220 OPG). On the other hand, there was an increase in FOC in the animals in the negative control group. This represents a statistically significant reduction in FOCR, which was highest ($p < 0.05$) for goats treated with 270 mg/Kg CNB in comparison to those treated with 90 mg/Kg CNB. In addition, after 28 days of treatment, the FOCR was 100% for the goats treated with 270 mg/Kg.

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Toxicity assessment of chitosan nanoencapsulated bromelain

Clinical observations

During the entire period of observation, the administration of 270 mg/Kg of CNB for 60 days did not show any mortalities or clinical signs in the group. The rectal temperature of the animal body varied between 37 to 38.5^{o}C and was within a normal range (Radostitis et al., 2000; Al-Bulushi et al., 2017) for the Small East African goat breed. Following the treatment, there were no significant differences in body temperatures ($p > 0.05$) in goats from different groups. The body weights of the goats before treatment were 25.00 ± 2.58 Kg (positive control Group), 16.50 ± 1.00 Kg (negative control), 16.25 ± 1.26 Kg (270 mg/Kg CNB), and 16.50 ± 1.00 Kg (90 mg/Kg CNB). After three weeks of treatment, the weight had increased to 18.88 ±1.93 Kg and 18.75 ±1.70 Kg of goats treated with 270 mg/Kg and 90 mg/Kg CNB, respectively, while the body weight decreased to 24.88±1.84 Kg for positive control (goats treated with Albendazole and Diclazuril) and to 16.13±1.08 Kg for negative control. The mean of body weights for the following treatment groups showed a significant ($p < 0.05$) increase (2.25 Kg) at the end of the study. This increase (compared to day 0) was statistically significant ($p < 0.05$). However, there was a decrease in weight by 0.62 to 1.62 Kg in goats of the positive control group ($p < 0.05$).

Effect of treatments on packed cell volume and biochemical parameters

Before treatment, the PCV levels were 32% (positive control group), 27.25% (negative control), 28.50% (270 mg/Kg CNB), and 28.25% (90 mg/Kg CNB). During the treatment, the PCV levels ranged between 25.25 to 30.00% for the goats treated with 270 mg/Kg and 90 mg/Kg CNB, 26.35 to 30.50% for positive control, and 18.25 to 26% for negative control. There were no significant differences ($p > 0.05$) in the PCV levels during the entire observation period.

During the treatment, AST ranged between 101 and 111 U/L for goats treated with CNB and between 115 and 121 U/L for the positive control group of animals. The ALT level was between 14.89 and 16.74 U/L and 16.51 and 17.93 U/L for goats treated with CNB and positive control, respectively. The creatinine level ranges were between 0.710 and 0.792 mg/L, while that for urea was between 33 and 35.06 mg/L for 270 mg/Kg and 90 mg/Kg CNB during the entire observation period.

Gross pathology and histopathological findings

No gross changes at necropsy were done on days 60 and 150 post first day of treatment. All the organs were normal and similar in both treatment and control Groups. The histopathological assessment showed no changes in the tissues in both groups treated with CNB (90 mg/Kg and 270 mg/Kg).

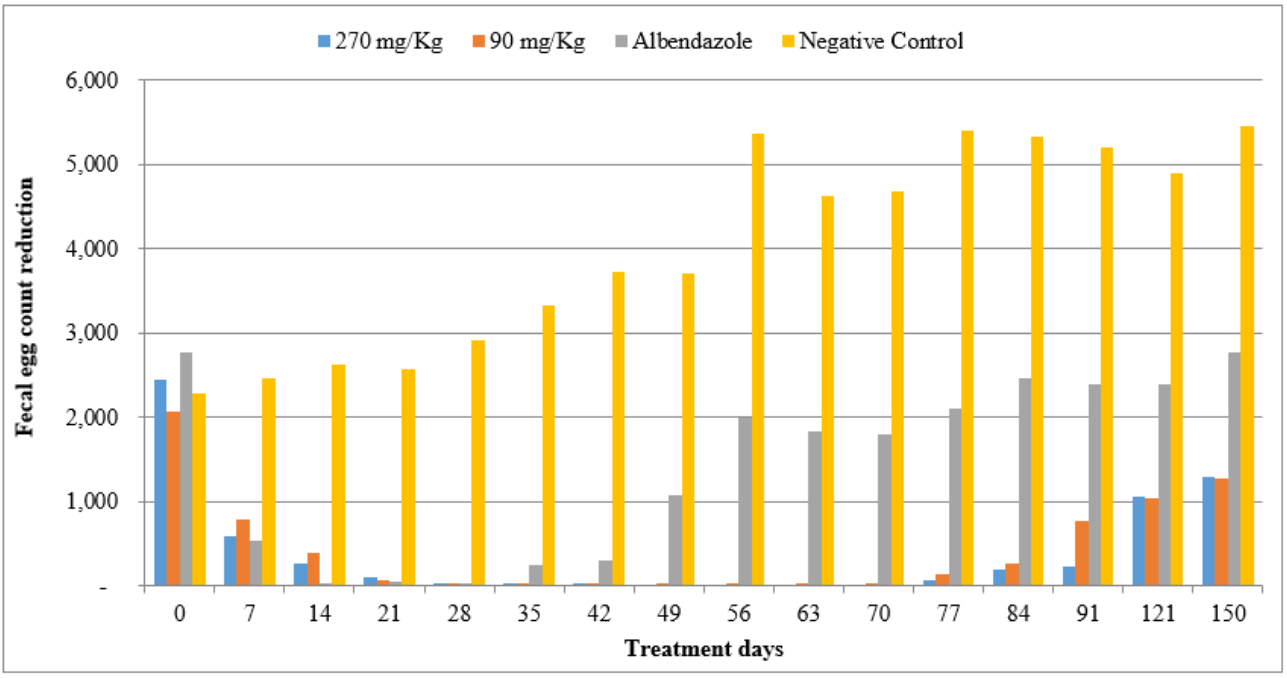

Figure 1. Fecal egg counts reduction in goats treated with chitosan nanoencapsulated bromelain (270 mg/Kg and 90 mg/Kg), Albendazole (7.5 mg/Kg), and untreated goats (negative control) in Kenya

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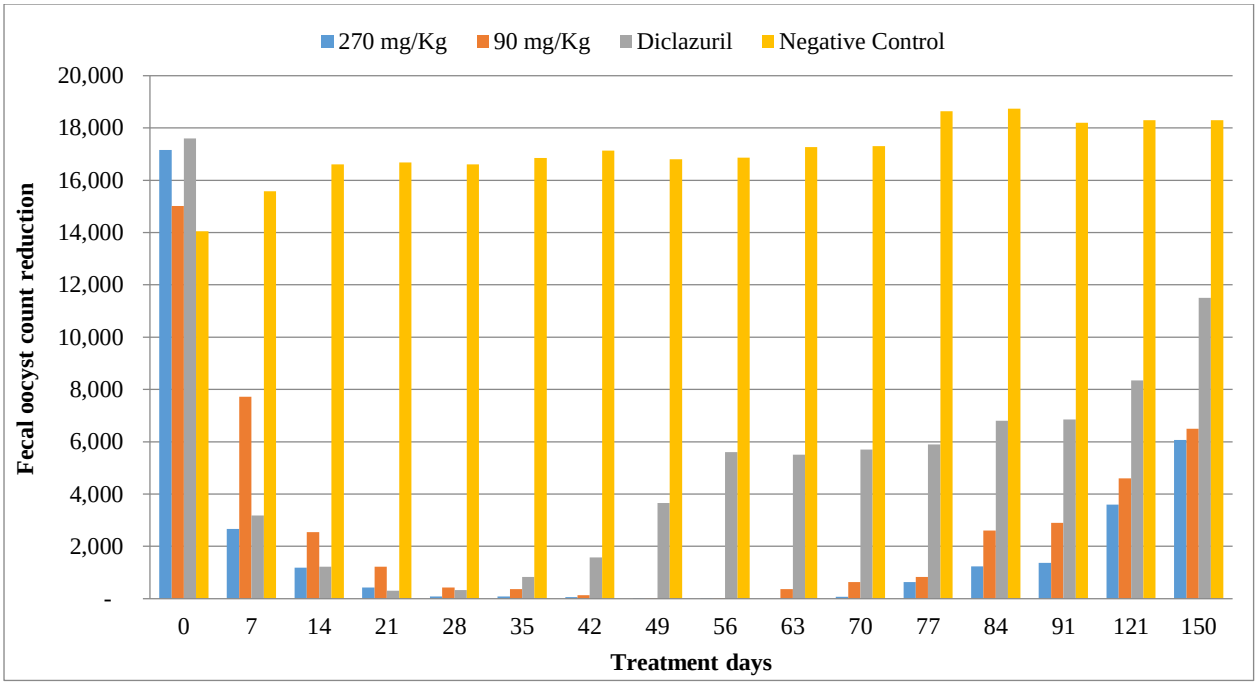

Figure 2. Fecal coccidial oocyst counts reduction in goats treated with chitosan nanoencapsulated bromelain (270 mg/Kg and 90 mg/Kg), diclazuril (5 mg/Kg), and untreated goats (negative control) in Kenya

DISCUSSION

The present study is a continuation of a previous investigation that evaluated the *in vivo* activity of chitosan nanoencapsulated bromelain (CNB) against GIN in Small East African goats in Kenya (Wasso et al., 2020). In the laboratory study, Wasso et al. (2020) observed an anthelmintic efficacy of 68.8% after administering CNB at 30 mg/Kg for 14 days. Administration of a single oral dosage of 90 mg/Kg and 270 mg/Kg CNB caused an efficacy of 9.5 % and 32.4%, respectively, in goats at 28 days post treatment (Wasso et al., 2020). The current study addressed the efficacy of CNB against natural infection of GIN and coccidia in goats. Present study results showed that administering CNB at higher doses considerably decreased the number of GIN eggs excreted by goats and reached 100% by 30 days post-treatment. Previous research on the anthelmintic action of pineapple (*Ananas comosus*) in sheep and goats has found that bromelain to have high efficacy against helminths (Hordegen et al., 2003; Domingues et al., 2013). However, when powdered leaves and plain bromelain were administered to sheep, Hordegen et al. (2003) and Domingues et al. (2013) reported a decrease in nematode eggs. The increased activity of CNB described by Wasso et al. (2020) and in the present study might be attributed to the encapsulation of bromelain with chitosan (Bhatnagar et al., 2014; Cheung et al., 2015). Nanoparticles, such as chitosan, boost the efficacy of drugs by avoiding enzymatic breakdown and increasing the drug's ability to access sites that would otherwise not be accessible to other drugs (Cheung et al., 2015). Bromelain, as previously described, induces cuticle deterioration and death of mature nematodes (Stepek et al., 2006; Behnke et al., 2008). Thus, the drop in egg excretion reported *in vivo* might be related to either adult worm death or a fall in female worm prolificacy. Stepek et al. (2005; 2006) found that papaya latex, which contains cysteine protease, significantly reduced worm load and egg yield in rodent GIN (*Trichuris muris* and *Heligmosomoides polygyrus*). Pascal et al. (2017) gave *Newbouldia laevis* (Bignoniaceae) to sheep and found it to be 55% effective against *H. contortus* and 19% effective against *T. colubriformis*. Hounzangbe-Adote et al. (2001) reported an 80% effectiveness rate of papaya (*Carica papaya*) seed powder on sheep *strongyles* after ten to fifteen days of therapy.

The present study showed that CNB significantly decreased the number of oocysts excreted by the goats to a level of efficacy comparable to the commercial drug (Diclazuril). This suggests that the enzyme is effective against coccidian infections as has also been reported elsewhere (Molan et al., 2009; Juasook et al., 2017; Dakpogan et al., 2019; Abdel-Tawab et al., 2020). Juasook et al. (2017) reported that after treating broilers chicken with crude pineapple extracts (*Ananas comosus*), *Eimeria tenella* oocyst production fell considerably ($p < 0.05$). On the other hand, Dakpogan et al. (2019) showed a 59% decrease in OPG in chicks fed with *Carica papaya* crude extracts. Bromelain has been shown to induce coccidia shell wall disintegration, softening, and loss of the core cytoplasmic mass (Juasook et al., 2017; Daiba et al., 2022).

The current study also evaluated the toxicity of CNB in goats. Similar to a previous study by Wasso et al. (2020), there were no mortalities or adverse clinical signs in the experimental animals. Further, the blood parameters such as PCV were within the species' normal range (Radostitis et al., 2000; Al-Bulushi et al., 2017), which implies that the

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longer administration duration was safe. The levels of AST, ALT, creatinine, and urea levels were also within the species' normal range (Radostitis et al., 2000; Jackson and Cockcroft, 2002). Similarly, there were no pathological lesions observed in the goats at postmortem, which agrees with the study by Wasso et al. (2020).

CONCLUSION

The current study found that CNB in chitosan exhibited high activity against GIN and coccidia infections in goats by lowering eggs and oocysts excretion. Further, CNB demonstrated no harmful effects on goats at repeated dosages of 270 mg/Kg. This sets the background and justification for further studies on strategies to improve efficacy through adjusting the dosage and duration of treatment with CNB. This should eventually lead to on-farm trials on the effectiveness of CNB against nematodes and coccidian. This could serve as validation ahead of a commercial venture to utilize this natural product as an alternative treatment of helminths and coccidian in small ruminant stock, but further study needs to prove this theory.

DECLARATIONS

Availability of data and materials

The data presented in this study are available by reasonable requests from the authors.

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Ethical consideration

All the authors have carefully examined all ethical issues concerning plagiarism, approval to publish, data fabrication and/or falsification, double publication, and submission.

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Competing interests

No competing interests.

Authors' contributions

All authors contributed to the conception of the experiments. Ahmota Romain Daiba, John Maina Kagira, and Maina Ngotho planned the study design. Ahmota Romain Daiba, James Kimotho, and Maina Ngotho performed sample collection and laboratory work. Naomi Maina conducted the data analyses. Ahmota Romain Daiba and John Maina Kagira wrote the draft of the manuscript. All authors corrected, read, and approved the final manuscript.

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Effects of Sumac (*Rhus coriaria*) Seeds and Exogenous Fibrolytic Enzymes on Wool Growth of Awassi Male Lambs

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ABSTRACT

Nutrition can have a significant effect on animal production. In recent years, many compounds have been widely used as feed additives to stimulate animals' appetites and consequently improve animal productivity. Exogenous fibrinolytic enzymes are one of these feed additives, which have been used as a digestive stimulant in different types of animals. Sumac (*Rhus coriaria*) seeds and leaves have been widely used as an appetite stimulant. Therefore, this study aimed to determine the dietary effects of using 0.3% exogenous fibrolytic enzymes and 3% of grinds *Rhus coriaria* seeds on wool production and some physical traits of the Awassi lambs. Twenty-four male Awassi lambs with an average age of 4 months were randomly assigned to four dietary treatments, each containing six animals. The control group received a basal diet equivalent to 2% of body weight. The second group received the same diet supplemented with 3% sumac (*Rhus coriaria*) powder. The third group received the basal diet supplemented with 0.3% exogenous fibrolytic enzymes (protease, amylase, and cellulase). The fourth group received the basal diet supplemented with both 3% *Rhus Coriaria* powder and 0.3% exogenous fibrolytic enzymes. The experiment lasted 130 days in the animal house belonging to the College of Veterinary Medicine in Iraq. Some wool traits, including wool staple length, clean wool weight, greasy wool weight, wool fiber length, and wool fiber diameter, were measured. The results revealed significant differences in all measured wool quality traits among the treated groups compared to the control group. The group receiving the diet supplemented with a combination of exogenous fibrolytic enzymes and *Rhus coriaria* powder exhibited the most significant improvements in wool growth, overall wool production, and physical characteristics. These findings highlight the potential of using exogenous fibrolytic enzymes and sumac as effective appetite stimulants and enhancers of wool production in Awassi lambs.

Keywords: Awassi Lambs, Fibrolytic enzymes, Staple, *Rhus Coriaria*, Wool

INTRODUCTION

Nutrition plays a significant role in determining wool characteristics and quality in sheep, along with genetic factors (Safari et al., 2005). The type of feed provided to sheep can influence wool production patterns (Holman et al., 2014). Wool growth is influenced by the availability of feed resources relative to the sheep's energy and maintenance requirements, which can vary in different conditions, such as during transportation over long distances or lactation periods (Holman and Malau-Aduli, 2013). Improved nutrition through enriched feeds has been associated with increased wool production, including thicker wool fiber diameter and enhanced wool growth, which are influenced by both genetics and nutrition (Jamshed Khan et al., 2012). Some medicinal plants feed additives such as sumac (*Rhus Coriaria*) have positive effects on the digestive system and are often good sources of some minerals, vitamins, and organic acids, such as malice, citric and tartaric acids. In addition, *Rhus coriaria* has antiviral, anti-inflammatory, anti-gastric disturbances, antioxidant, antibacterials, and antidiarrheal effects (Duke et al., 2003; Khayatnouri et al., 2011).

Feed additives are commonly used in animal diets to improve metabolism and enhance palatability (Wafar et al., 2023) Some feed additives added are commonly used in animal diets to improve metabolism and enhance palatability (Khudhair and Al-Saadi, 2022; Wafar et al., 2023). These feed additives are frequently used in ruminants' diets to enhance digestion metabolism rate and are also used as growth promoters (Beauchemin et al., 2001). Some investigators revealed that fibrolytic enzymes, such as protease, cellulose, and xylanase increase, have been found to improve nutrient digestion and utilization as well as mitigate the negative effects of some types of feed certain feeds in both ruminants and monogastric animals (Salem et al., 2013). In normal cases, ruminal microflora produces sufficient amounts of enzymes that have important benefits for improving digestibility and enhancing animal performance by increasing feed consumption and feed conversion efficiency (Wang et al., 2012). So, the main objective of the current study was to

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investigate the effects of feeding Awassi male lambs a diet supplemented with 0.3 % of% exogenous fibrolytic enzymes, including (Protease, Cellulase, and Amylase), and 3% (*Rhus coriaria*) ground sumac seeds, (*Rhus coriaria*), mixed or separately to the diet of the Awassi male lambs on their wool production and some of its physical characteristics (Khudhair and Al-Saadi, 2022). These feed additives are frequently used in ruminants' diets to enhance digestion metabolism rate and are also used as growth promoters (Beauchemin et al., 2001). Fibrolytic enzymes such as protease, cellulase, and xylanase have been found to improve nutrient digestion and utilization while mitigating the negative effects of certain feeds in both ruminants and monogastric animals (Salem et al., 2013). In normal cases, ruminal microflora produces sufficient amounts of enzymes that have important benefits for improving digestibility and enhancing animal performance by increasing feed consumption and feed conversion efficiency (Wang et al., 2012). So, the main objective of the current study was to investigate the effects of supplementing the diet of Awassi male lambs with 0.3% exogenous fibrolytic enzymes (Protease, Cellulase, and Amylase), and 3% ground sumac seeds (*Rhus coriaria*), mixed or separately to the diet of the Awassi male lambs on their wool production and physical characteristics**.**

MATERIALS AND METHODS

Ethical approval

The design and procedure of the current experiment were approved by the Scientific Research Committee of the Department of Public Health College of Veterinary Medicine (Iraq) for approval of the experimental protocols at the annual scientific meeting of Baghdad University on 12-2-2021 with project number (PNR/FSM/12/2/2021).

Study period and location

The current research was performed on a farm belonging to the College of Veterinary Medicine, University of Baghdad, Iraq. The experimental duration was 130 days, including 10 days adaptation period, from January to May 2021.

Ingredients of the diet

Table 1 shows the basal diets used in feeding Awassi sheep during the study with their chemical contents formulated according to the Nutrient Requirements of Beef Cattle**.**

Table 1. The ingredients and chemical composition of the diet for Awassi sheep in Iraq

Content of dry matter (gm /Kg)	
Barley grain	720
Wheat bran	140
Soya meal	120
Dicalcium phosphate*	10.5
Limestone	4.50
Vitamins - minerals mix	1.25
Salt	2.25
Sodium bicarb.	1.50
Chemical composition (DM %)	
OM	84.5
NFE	57.5
CP	16.5
ADF	24.5
NDF	53.5
CF	23.5
Ash	12.0
EE	1.8
Energy (MJ/Kg DM)	26.5

CP: Crud protein- TDN: Total digestible nutrient- NDF: Neutral detergent-fiber- ADF: Acidic detergent fiber- NFE: Nitrogen- free extract- EE: Ether extract-CF: Crud fiber- DM: Dry matter-OM: Organic matter- Dicalcium phosphate*- : 28% Ca, 19%P-(NRC, 2000).

Experimental design

A total of 24 Awassi male lambs, averaging 4 months in age and weighing 20 ± 0.5 kg, were included in this study. The lambs were provided with a concentrated basal diet (Table 1) and allowed to freely graze on Alpha-Alpha plants for 2-4 hours daily. Lambs were randomly divided into four dietary treatments, each consisting of six animals. To ensure accurate individual data collection and minimize experimental error, each treatment group was housed in a separate pen measuring 2.5 m × 2 m during feeding. The diet was formulated every 2 weeks. The first group was fed a 2% of body

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weight basal diet and regarded as the control group. The second group was fed the same percentage containing 3 % *Rhus coriaria* powder. The third group was fed the same percentage of basal diet per head containing 0.3% of exogenous fibrolytic enzymes, including Protease, Amylase, and Cellulase enzymes (Bioagripharm GmbH-56564-Germany, batchNo.21664). The fourth group fed the same percentage a head containing 3 % *Rhus coriaria* powder and 0.3%of exogenous fibrinolytic enzymes (Ikusika et al., 2019).

Sampling

Three sampling periods were conducted between January 1 and May 10, 2021, with 2.5 months intervals. The first sample was taken at the beginning of the experiment on day 1, the second sample at day 75, and the third sample at day 130. Samples were collected from all sheep in the experimental groups. A 5x5 cm² area of wool was sheared from the right chest's last ribs to measure wool growth and physical characteristics such as greasy wool weight, clean wool weight, wool staple length, fiber length, and fiber diameter (Al-Saadi et al., 2012; Al-Saadi and Al-Zubiadi, 2016).

Statistical analysis

All results were documented using Microsoft Office Excel and analyzed statistically using the SPSS software, version 24. Data were analyzed with a complete randomized design method, and the Least Significant Differences (LSD). The level of significance was considered $p < 0.05$ to identify the differences between different groups (Chemezova and Zaykov, 2014).

RESULTS

According to Table 2, there was a significant ($p < 0.05$) increase in greasy wool weight for groups G2, G3, and G4 during the second and third sampling periods compared to the control group. However, there were no significant differences among the groups during the first sampling period. Similarly, clean wool weight followed a similar trend (Table 3). Group G4, which received a combination of feed additives, showed significantly higher levels, compared to both the *Rhus coriaria* and Exogenous fibrinolytic enzymes groups individually, as well as the control group ($p < 0.05$). These differences were particularly pronounced in the last two sampling periods. However, there were no significant differences between the groups during the first sampling period

The weight increase of wool fibers in all treated groups is closely related to the increase in wool fiber length, staple length, and wool fiber diameter. These changes were evident in the results presented in Tables 4, 5, and 6. All treated groups showed a significant increase ($p < 0.05$), with the mixed group (G4) demonstrating the highest levels compared to both the *Rhus coriaria* and exogenous fibrolytic enzymes groups, particularly during the third sampling period. The control group exhibited significantly ($p < 0.05$) lower levels, compared to all treated groups, especially during the last two sampling periods. However, there were no significant differences between the groups in the first sample.

Table 2. Effects of *Rhus coriaria* seed and exogenous fibrolyic enzymes on greasy wool weight (g) of Awassi male lambs in Iraq

Group	Control group	*Rhus coriaria* group	Enzymes group	Mixed group	LSD
First sampling	1.87 ± 0.94^{a}	1.86 ± 0.32^{a}	1.85 ± 0.42^{a}	1.84 ± 0.24^{a}	0.66
Second sampling	0.55 ± 0.73^{c}	0.93 ± 0.41^{b}	0.82 ± 0.67^{b}	1.10 ± 0.11^{a}	0.15
Third sampling	2.11 ± 0.45^{d}	2.68 ± 0.24^{b}	2.34 ± 0.47^{c}	2.84 ± 0.31^{a}	0.14

^{A,b,c} superscript letters in the same row means significant differences within groups at $p < 0.05$. The data expressed as means ± SE

Table 3. Effects of *Rhus coriaria* seed and fibrolyic enzymes on clean wool weight of Awassi male lambs (g) in Iraq

Group	Control group	*Rhus coriaria* group	Enzymes group	Mixed group	LSD
First sampling	1.47 ± 0.15^{a}	1.46 ± 0.33^{a}	1.48 ± 0.81^{a}	1.46 ± 0.21^{a}	0.25
Second sampling	0.50 ± 0.43^{c}	0.91 ± 0.75^{a}	0.75 ± 0.36^{b}	1.05 ± 0.44^{a}	0.15
Third sampling	1.65 ± 0.73^{c}	2.05 ± 0.54^{b}	1.98 ± 0.45^{b}	2.22 ± 0.15^{a}	0.14

^{A,b,c} superscript letters in the same row means significant differences within groups at $p < 0.05$. The data expressed as means ± SE

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Table 4. Effects of *Rhus coriaria* seed and fibrolyic enzymes on wool fibers length (cm) of Awassi male lambs in Iraq

Group	Control group	*Rhus coriaria* group	Enzymes group	Mixed group	LSD
First sampling	7.35 ± 0.57^{a}	7.92 ± 0.75^{a}	7.38 ± 0.61^{a}	7.74 ± 0.82^{a}	0.31
Second sampling	4.34 ± 0.22^{c}	4.99 ± 0.34^{b}	4.70 ± 0.74^{b}	5.41 ± 0.74^{a}	0.26
Third sampling	5.40 ± 0.13^{c}	6.08 ± 0.54^{b}	5.93 ± 0.36^{b}	6.89 ± 0.74^{a}	0.22

^{A,b,c} superscript letters in the same row means significant differences within groups at $p < 0.05$. The data expressed as means ± SE

Table 5. Effects of *Rhus coriaria* seed and fibrolyic enzymes on wool staple length (cm) of Awassi male lambs in Iraq

Group	Control group	*Rhus coriaria* group	Enzymes group	Mixed group	LSD
First sampling	7.38 ± 0.24^{a}	7.22 ± 0.32^{a}	7.27 ± 0.12^{a}	7.41 ± 0.78^{a}	0.33
Second sampling	4.40 ± 0.17^{c}	4.89 ± 0.54^{b}	4.72 ± 0.31^{b}	5.21 ± 0.44^{a}	0.25
Third sampling	5.22 ± 0.34^{d}	5.72 ± 0.24^{c}	6.70 ± 0.19^{b}	6.92 ± 0.22^{a}	0.17

^{A,b,c,d} superscript letters in the same row means significant differences within groups at $p < 0.05$. The data expressed as means ± SE

Table 6. Effects of *Rhus coriaria* seed and fibrolyic enzymes on wool fiber diameter (µ) of Awassi male lambs in Iraq

Group	Control group	*Rhus coriaria* group	Enzymes group	Mixed group	LSD
First sampling	29.15 ± 0.35^{a}	28.72 ± 0.25^{a}	28.65 ± 0.33^{a}	28.84 ± 0.15^{a}	0.38
Second sampling	30.21 ± 0.39^{c}	31.25 ± 0.14^{b}	31.54 ± 0.24^{b}	32.11 ± 0.30^{a}	0.15
Third sampling	31.54 ± 0.43^{d}	33.25 ± 0.58^{b}	32.65 ± 0.48^{c}	34.36 ± 0.30^{a}	0.46

^{A,b,c,d} superscript letters in the same row means significant differences within groups at $p < 0.05$. The data expressed as means ± SE

DISCUSSION

The results of the present study showed a significant difference regarding all investigated variables in all groups compared to the control group ($p < 0.05$), particularly in the group supplemented with 3% *Rhus coriaria* and 0.3% exogenous fibrolytic enzymes during the last two sampling periods. These positive effects can be attributed to the synergistic effects of both feed additives. It is likely that these additives acted as appetite enhancers and had high levels of vitamin C (Ascorbic acid), especially in *Rhus coriaria*, which is known for its antioxidant properties. Additionally, the exogenous fibrolytic enzymes improved rumen digestibility by activating microflora (Al-Saadi and Mohammed, 2022). These findings are consistent with previous studies conducted by various researchers (Rowe et al., 1989; Masters et al., 1998; Al-Saadi et al., 2012). These authors reported that some feed additives act as appetite promotors, and have positive effects wool growth and the quality of wool (Al-Saadi et al., 2012). nThe positive changes observed in this study could be attributed to increased blood circulation in the skin, leading to improved nutritional supply. This improvement is influenced by the type and nutritional value of the diet supplementation (Jamshed Khan et al., 2012). However, the levels of wool growth can vary depending on the sheep genotype, and is influenced by numerous physiological and dietary factors (Malau-Aduli et al., 2019). This suggests that feeding medicinal plants, such as *Rhus coriaria*, to animals can have stimulatory effects on their digestive system, which are considered a good source of protein, minerals, vitamins, as well as antioxidants, antibacterial, and antidiarrheal compounds (Reis et al., 1992). According to Malau-Aduli et al. (2019), the positive improvements in wool production and quality can be attributed to the increased absorption of specific amino acids, such as cysteine. Additionally, wool growth in certain sheep breeds, like Merino, responds to changes in nutrition throughout the year. Increasing essential nutrients during the appropriate seasons can lead to an increase in the rate of wool growth (Kott et al., 1999; Allden, 1979) reported the improvement of rumen digestive action as a result of sumacs and exogenous fibrinolytic enzymes supplementation (Kott et al., 1999). Furthermore, several authors have reported that supplementation of sumacs and exogenous fibrolytic enzymes can enhance rumen digestion (Kott et al., 1999). Similarly, numerous studies have demonstrated improved digestibility of fibrous diets in ruminants through the use of various biotechnological products such as direct-fed microbes, ionophores, and cell wall degrading enzymes. By supplementing fibrinolytic enzymes and incorporating sumacs as appetite promoters in the diets, viable rumen microflora and the growth and movement of ruminal microorganisms can be increased. This, in turn, leads to greater absorption of amino acids and minerals through the intestinal mucosa, thereby significantly influencing the metabolic protein resource available to the animal's tissues (Reis et al., 1992). Therefore, these feed additives that are supplemented in the diet of livestock can improve levels of wool growth production by an increase in protein rates and mineral resources (Nsereko et al., 2002; Al-Saadi and Al-Zubiadi, 2016).

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It is noteworthy that these results are consistent with findings from other studies, where researchers observed clear improvements in the digestibility of neutral detergent fiber, organic matter, dry matter, and acid detergent fiber in sheep fed a diet enriched with fibrolytic enzymes. These improvements ultimately contributed to enhanced wool production overall (Titi and Tabbaa, 2004). However, data obtained from the current study revealed that the effects of *Rhus coriaria* seeds were more significant, compared to exogenous fibrolytic enzymes in the last two sampling periods and approximately in all parameters. This could be attributed to the appetite-enhancing properties of *Rhus coriaria*, as well as its rich nutrient content, including vitamins, minerals, and antioxidant substances. These findings align closely with previous studies. Jafari et al. (2004) and Allden (1979) reported similar observations, highlighting a positive linear correlation between feed intake, dry matter, and wool growth. In contrast to the present results, some researchers have reported no correlation between nutrient values and wool production (Moioli et al., 2015). On the other hand, other studies have suggested that wool growth levels are influenced by changes in body weight as a result of overall body growth (Kott et al., 1999; Al-Saadi and Al-Zubiadi, 2016). These findings have indicated that the effects of weight changes on wool production levels and the relationship between energy and wool growth remain unclear and require further in-depth investigation (Jafari et al., 2004; Titi and Tabbaa, 2004). Some researchers have determined that high level of wool production is related to an increase in dry matter intake (Malau-Aduli et al., 2019). This increase represented the increase in fiber size created in normal feed levels (Reis and Panaretto, 2001). Moreover, the present study aligns with other researchers who have suggested that wool quality and growth depend on the type of protein added to the diet and the amount provided (Masters et al., 1998). Certain types of protein are rich in amino acids, particularly cysteine, which significantly influence follicular uptake and wool fiber production (Li et al., 2008). Therefore, an increase in amino acid levels, especially those containing sulfur, leads to enhanced protein availability and improved nutrient uptake by wool follicles (Malau-Aduli and Holman, 2010; Malau-Aduli et al., 2019).

CONCLUSION

The positive effects of supplementing the diet of Awassi sheep with both sumacs and exogenous fibrolytic enzymes have been observed in terms of enhanced wool production and improved physical quality. These effects were observed when the additives were used separately or in combination. Based on these findings, it is recommended to further investigate the effects of seasons and different percentages of these feed additives. Additionally, studying the effects of supplementing minerals on wool production could also be beneficial.

DECLARATIONS

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Author's contributions

M.J.Al-Saadi developed the idea and conducted the laboratory work writing, editing, and creation of the final draft. The author read and approved the final draft.

Competing interests

The authors have not declared any conflict of interest.

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Availability of data and materials

All data are available on request.

Ethical consideration

The authors take steps to abide by all ethical standards related to plagiarism, publication approval, inaccuracies in data, multiple submissions, and double publication.

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Semen Cryopreservation Quality and Sperm Kinematics of Saanen Goats Using Different Diluents

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ABSTRACT

The success of artificial insemination (AI) in small ruminants, especially goats, depends on the quality of frozen semen. Therefore, the current study aimed to determine the quality of various diluents, including tris-egg yolk, AndroMed®, and OviXcell®, on semen quality. The fresh semen samples from three male Saanen goats aged 1.5-2 years were collected and the mean individual motility of samples was recorded at 70%. The cryopreservation quality of the semen was evaluated based on motility, viability, abnormality, and total sperm motility (TSM) indexes. The present laboratory experiment was performed with 3 treatments and 10 repetitions. The treatments in this study were T0 (tris-egg yolk), T1 (AndroMed®), and T2 (OviXcell®). The results showed no significant difference in the parameters of motility, viability, abnormality, and TSM among the treatment group. The kinematic parameters' average path length, velocity curved linear, and linearity showed a significant difference in all treatment groups. However, there were no significant differences among the three groups in terms of motility, progressiveness, distance curved line, distance straight line, average velocity path, velocity straight line, straightness, amplitude lateral head, beat cross frequency, and wobble kinematic parameters. Motility was higher in T2 than in T0 and T1, viability was higher in T1 than in T0 and T2, and abnormality was lower in T1 than in T0 and T2. In conclusion, the use of various diluents, such as tris-egg yolk, AndroMed®, and OviXcell®, can maintain the quality of frozen spermatozoa for over 24 hours, including motility, viability, abnormality, and TSM. Kinematic parameters obtained using CASA IVOS II can provide relevant information for various parameters using these diluents.

Keywords: Computerized Assisted Sperm Analyzer, Goat's sperm, Saanen goats, Semen quality, Sperm cryopreservation

INTRODUCTION

Saanen goats are a type of dairy goat originating from Switzerland with the primary product of milk (Susilorini, 2019). Male Saanen goats have higher levels of calcium, sodium, phosphorus, and magnesium, compared to females, making them superior in terms of nutrient content (Vargas et al., 2018). Saanen goat is a popular breed for milk production and can produce an average of 750 kg of milk, making them adaptable to various regions of the world (Sadjadian et al., 2012). The adaptability of Saanen goats and their high milk production make them a promising area for further research.

Since the origin of the Saanen goats is in subtropical regions, additional research is necessary to improve their management for optimal productivity in Indonesia (Anggraeni et al., 2020). Productivity improvement can be optimized by improving genetic quality. Genetic quality improvement in livestock can be done by applying Artificial Insemination (AI). Artificial insemination is a reproductive technology that is often successful and widely accepted by the wider community, especially breeders (Rege et al., 2011). The AI is more widely applied in large ruminants than in small ruminants. This is in accordance with previous studies indicating a success rate of 47.05% for AI in small ruminants, especially dairy goats (Ciptadi et al., 2014). Factors that influence the success of AI include livestock conditions, feed, environmental conditions, semen quality, and inseminator skills.

In Indonesia, frozen semen is commonly used for AI in goats. The production of frozen semen involves several stages, including preparation, semen storage, semen evaluation, dilution, packaging, freezing, and inspection after freezing (Moore and Hasler, 2017). The success of freezing semen largely depends on the type of diluent used. The process of producing frozen semen requires a diluent to maintain the quality of semen (Arif et al., 2022). Diluents are added to semen to protect and energize the spermatozoa and prevent cold shock during cryopreservation (Tethool et al., 2021). The most commonly used diluents for freezing semen in Indonesia are tris-egg yolk, AndroMed®, and OviXcell®. Egg yolk is added as an extracellular cryoprotectant and energy source, containing lecithin and lipoprotein (Tethool et al., 2022). To determine the effectiveness of semen dilution, it is important to examine the quality of the

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diluent used. The high-quality diluent can protect the membrane cell and prevent cold shock (Arif et al., 2022). Cold shock occurs due to adaptation during storage and gradual cooling from 30°C to 4°C for 2 hours (Falchi et al., 2018).

Semen used for insemination must be tested for its quality. Semen quality tests can be performed at macroscopic and microscopic stages (Arif et al., 2020). The quality analysis of goat semen during freezing is carried out to determine the frozen semen's suitability for insemination. Parameters of motility, viability, and abnormality are usually used to determine the quality of goat semen during storage (Davendra and Krishnappa, 2019). The motility of sperm can be measured visually or using Computerized Assisted Sperm Analyzer (CASA). Motility assessment results with CASA are more objective, accurate, repeatable, and standardized. The CASA is based on the movement and trajectory of spermatozoa (Anand and Yadav, 2016). The aim of this study was to analyze the quality of the frozen semen of Saanen goats using different diluents, namely tris-egg yolk, AndroMed®, and OviXcell®.

MATERIALS AND METHODS

Ethical approval

The research was conducted at the Singosari Center for Artificial Insemination (SCAI), Malang, East Java, Indonesia, according to standard procedures of SNI ISO 9001:2015 NO.G.01-ID0139-VIII-2019 and supervised by veterinarians. Ethical guidelines and approvals on research procedures have been provided by the SCAI ethics committee. This research has been approved by the ethics committee of the University of Brawijaya number 009-KEP-UB-2023.

Study design

The current study was performed on three male goats aged 1.5-2 years from the Singosari Center for Artificial Insemination (SCAI) Singosari, Malang, East Java, Indonesia. The fresh semen samples Saanen goats aged 1.5-2 years. The goats were kept in a pen at a temperature of 28-30°C under normal conditions. The goat pen had enough lighting using an intensive system with grazing in the paddock in the morning. Each goat had its own pen with a size of 2.5 x 2 m^2. The feed composition consists of 2 kg/head of forage, 2.5 kg/head of legumes, and 1 kg/head of concentrate. A laboratory experimental method was used for this study with 3 treatments with 10 repetitions. The three goats had body weights of 32.5 kg, 43 kg, and 44.5 kg. The semen aseptically was collected once a week for 10 weeks using an artificial vagina (IMV, France). A teaser was used to induce libido and facilitate semen collection.

Diluent preparation

The dilution process involves the use of three different diluents, namely tris-egg yolk (T0), AndroMed® (T1), and OviXcell® (T2). The T0 was made up of1.363 g tris aminomethane (Merck, Germany), 0.762 g citric acid (Serva, USA), 15 g lactose (Serva, USA), 0.5 g fructose (Kanto Chemical, Japan), 20 g egg yolk, 2.7 g raffinose (Serva, USA), 0.1 g streptomycin (Meiji, Indonesia), 80 ml aquabidest 0.1 g penicillin (Meiji, Indonesia), and 13% glycerol (Merck, Germany). The T1 using AndroMed® (Minitube, Germany) contained aquabidest, fructose, glycerol, citric acid, buffer, phospholipids, spectynomycine, 15 mg lyncomycine, 5 mg tylocin, and 25 g gentamycin (Susilawati, 2011). Finally, T2 entailing OviXcell® (IMV, France) had ultra-pure water, salt, sugar, electrolytes, glycerol, antibiotics protein, and some ingredients that were not listed by the manufacturer (Kakati et al., 2019).

The tris-egg yolk diluent was divided into three parts, namely volume A, volume B, and volume C. Volume A had no glycerol with a ratio of volume and semen of 1:1. Volume B, without glycerol, was added after the semen has equilibrated at 4-5°C and volume C contained 13% glycerol. AndroMed® was diluted with aquabides and could be used immediately in a ratio of 1:4. For example, 20 ml of AndroMed® was added to 80 ml of aquabides (in a 100 ml preparation). The semen mixed with AndroMed® was immediately put in the cool top until the temperature reached 4-5°C, then the dilution process with semen continued. OviXcell® did not require any additional dilution with aquabides before being mixed with semen. It could be directly mixed with semen without the need for any intermediary dilution step. The dilution formulas applied to Singosari Center Artificial Insemination for frozen semen are as follows (Shukla, 2020).

$$\text{Total volume} = \frac{\text{Semen Volume} \times \text{Concentration} \times 10^6}{200 \times 10^6} \qquad \text{Formula 1 (All diluents)}$$

$$\text{Volume A} = 1{:}1 \text{ (semen: diluent)} \qquad \text{Formula 2 (All diluents)}$$

$$\text{Volume B} = \frac{\text{Total Volume}}{2} - \text{Volume A} \qquad \text{Formula 3 (All diluents)}$$

$$\text{Volume C} = \frac{\text{Total Volume}}{2} \times \text{glycerol } 13\% \qquad \text{Formula 4 (tris-egg yolk diluent)}$$

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To maintain semen quality during cold storage, it was important to dilute the semen using a water bath with a temperature of 37°C and then stored it at 4-5°C. The diluent and glycerol conditions must be the same as the treatment so that the semen quality during cold storage can be maintained. In order to proceed to the freezing stage, the semen must be of high quality. If the motility of the semen before freezing is less than 55%, which is the minimum acceptable standard according to the Indonesian National Standard (2014), it should be rejected.

Semen collection

Semen was collected once a week in the morning at 10 using an Artificial Vagina (IMV, France) filled with 450-500 ml of 50-60°C warm water. The artificial vagina was pumped to a certain consistency and coated with lubricating jelly. The volume of Saanen goat's semen obtained was around 0.5-1 ml. The collected semen was immediately brought to the research site for semen dilution. The minimum dose of spermatozoa in diluent was 200 million sperm cells in each tube at the time of the study.

Cryopreservation and semen dilution procedures

After carrying out the shelter of semen, a quality test was carried out before dilution. This test included analyzing the motility, viability, abnormality, and total sperm motility (TSM) of fresh, liquid, and frozen semen using a radiance microscope (Olympus BX-53, Japan) with 400x magnification. After freezing, a more detailed test was carried out by observing kinematic parameters using a Computerized Assisted Sperm Analyzer (CASA, Hamilton Thorne, USA). Cryopreservation of goat semen using cold storage and length of time according to the type of diluent, tris-egg yolk was diluted at a ratio of 1:1 with a diluent temperature of 37°C (Susilawati, 2011). Equilibration was carried out for 2 hours (Volume A), then Volume B was added and stored for 18-22 hours at 4-5°C for tris-egg yolk diluent (Tethool et al., 2022) before adding 13% glycerol (Volume C). For AndroMed® and OviXcell® diluent process of filling and sealing was immediately carried out if the test of the quality of the liquid semen was decent. At the time of freezing, a 0.25 ml straw was filled with semen and then stored in liquid nitrogen (N_2) at a temperature of -196°C.

Statistical analysis

The obtained data were analyzed using SPSS software (version 29 IBM) with one-way ANOVA and a follow-up Duncan Multiple Range Test (DMRT) when the p-value was significant ($p < 0.05$). All data are presented as a mean ± standard error (SE).

RESULTS AND DISCUSSION

Semen cryopreservation in goats involves the use of cooling and freezing methods to dilute and preserve spermatozoa. However, it can decrease sperm quality due to temperature changes during storage and freezing (Memon et al., 2012), the formation of ice crystals, and changes in electrolyte concentrations (Tekin and Daşkin, 2019). It was stated that during the cooling and freezing phases, this damage can result in the production of ROS, lack of energy, ionic imbalance, changes in cell volume, hyperosmolarity, and protein denaturation (Ugur et al., 2019). To prevent this, a semen diluent is required to maintain the quality of the spermatozoa during cryopreservation.

The motility results showed no significant difference in semen motility of the treatments ($p > 0.05$). However, the lowest motility percentage was obtained in T0 and the highest motility percentage was indicated in T2 (Table 1). Motility is the power or movement of spermatozoa that moves forward or progressively. Motility is an important factor in determining sperm viability and its ability to fertilize an oocyte (Setiyono et al., 2020). As indicated in Table 2, the highest motility was obtained in T2 (59 ± 1.00), while the lowest motility was in T0 (49.50 ± 2.29). The decrease in motility in T0 during thawing may be due to the freezing process, which can cause damage to spermatozoa. The addition of egg yolk into the freezing extender increases the proportion of motile sperm (Aboagla and Terada, 2004). The results obtained in T2 were greater than those of Kakati et al. (2019) at 36 ± 1.21. However, the results of the T0 study were lower than those reported by Crespilho et al. (2012) at 58.52 ± 2.98. The difference in the percentages of T0 and T2 was due to the different diluents used, namely tris-egg yolk and OviXcell®. Tris-egg yolk contains egg yolk and OviXcell® contains soy lecithin so it has a different lipid composition and fatty acid content (Roof et al., 2012). Tris-egg yolk has a lower boiling point (<100°C) than the boiling point of tris solution (100°C), resulting in the formation of small granules of fructose during observation and consequently low motility (Susilawati, 2011). On the other hand, the fast progressive movement of OviXcell® diluent is because it contains soy lecithin and the diluent has a patent based on the original composition of the manufacturer (Kakati et al., 2019). OviXcell® contains soy lecithin can reduce damage due to cold shock during the freezing and thawing process (Nadri et al., 2019). As stated, the addition of 1% soy lecithin was successful in the cryopreservation of goat semen (Salmani et al., 2014). In the same line, Vidal et al. (2013) reported that the addition of soy lecithin at 0.04%, 0.08%, and 0.16% could be effective in freezing goat semen.

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Table 1. Semen quality of Saanen goats after freeze-thawing in various diluents

Parameters \ Treatment (Mean ± SE)	T0	T1	T2
Motility (%)	41 ± 2.87	46 ± 4.64	48.5 ± 2.89
Viability (%)	65.25 ± 8.09	67.53 ± 3.34	59.22 ± 5.54
Abnormality (%)	4.02 ± 1.34	1.87 ± 0.38	3.21 ± 0.41
TSM (million/straw)	16.75 ± 1.83	15.50 ± 1.82	17.25 ± 1.69

T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®

Table 2. Semen cryopreservation quality of Saanen goats before and after freezing

Parameters	Fresh semen	Semen quality before freezing (Mean ± SE)			Semen quality after freezing (Mean ± SE)		
		T0	T1	T2	T0	T1	T2
Sperm Motility (%)	73.5 ± 2.42	49.50 ± 2.29	55 ± 2.36	59 ± 1.00	41 ± 2.87	46 ± 4.64	48.50 ± 2.89
Sperm Viability (%)	72.69 ± 11.12	63.08 ± 6.84	79.41 ± 3.46	74.59 ± 4.00	65.25 ± 8.09	67.53 ± 3.34	59.22 ± 5.54
Sperm Abnormality (%)	2.78 ± 2.36	2.32 ± 0.76	2.4 ± 0.79	2.27 ± 0.49	4.02 ± 1.34	1.87 ± 0.38	3.21 ± 0.41
Total Sperm Motility (million/straw)	2706.90 ± 818.71	45.00 ± 1.15	50.00 ± 1.18	52.73 ± 0.41	16.75 ± 1.83	15.50 ± 1.82	17.25 ± 1.69

T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®

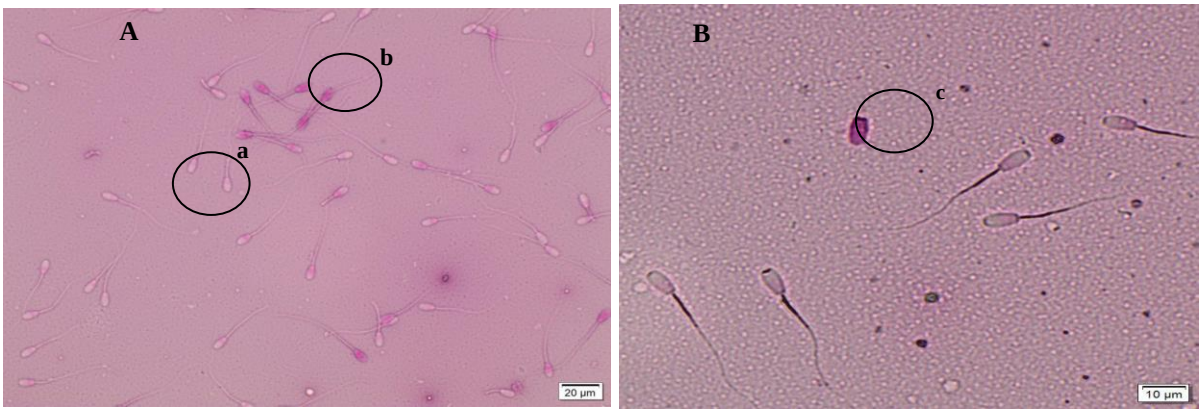

Figure 1. The viability and abnormal spermatozoa after thawing of the Saanen goats (eosin-nigrosine, x 400 magnification). **A**: Viable (the head of spermatozoa does not absorb [a]), Nonviable (b); **B**: abnormality of sperm (c).

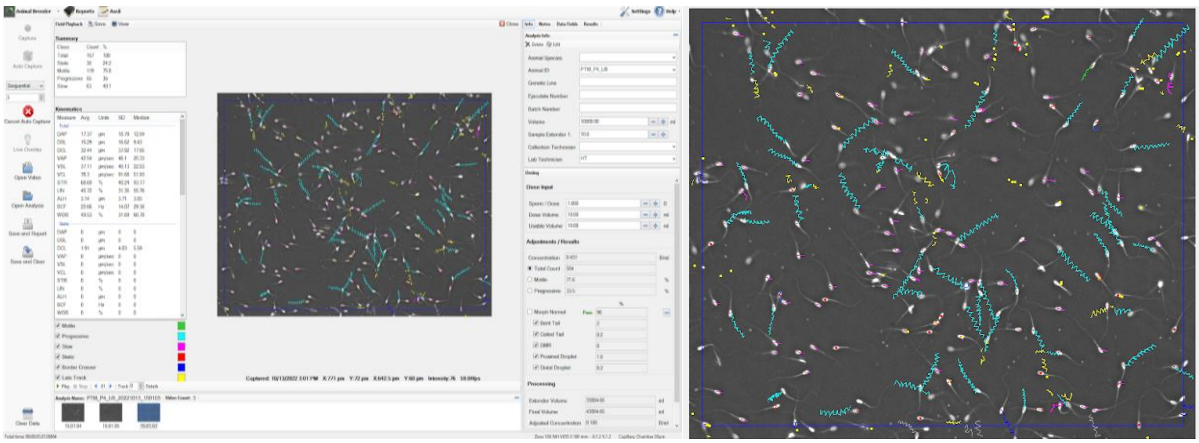

Figure 2. The kinematic parameters using CASA IVOS II (Hamilton Thorne, USA) with information green: Motile, aqua: Progressive, pink: Slow, red: Static, blue: Border crosser, yellow: Late track

The viability or survival of spermatozoa can be determined by assessing their ability to absorb the eosin-nigrosine stain on spermatozoa (Susilawati, 2011). Viability can be determined by mixing semen with eosin-nigrosine in a ratio of 10:10 µl (Yodmingkwan et al., 2016). Based on the results of the study, there was no significant difference in the viability of spermatozoa among the treatments ($p > 0.05$). However, the highest percentage of viability was observed in T1 and the lowest was in T2 (Table 1). The outcomes of this research are presented in Figure 1, where dead spermatozoa appear stained with pigment, while live spermatozoa do not absorb the stain. The viability of semen decreases during the process of cryopreservation, which is due to the storage of liquid semen in a frozen state (Shah et al., 2022). This

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decrease in viability is caused by damage to the cell membrane, which is a complex composition including two layers of phospholipids, proteins, glycoproteins, and carbohydrates (Peris-Frau et al., 2020). This is caused by a decrease in temperature during the cold storage process, which causes the lateral movement of phospholipids to change between the liquid and frozen phases leading to the membrane being stiff and brittle (Card et al., 2013). As can be seen in Table 2, viability in T1 was (79.41 ± 3.46), which was higher than in T0 (63.08 ± 6.84) before freezing. This was caused by the lecithin content in the T1 diluent, which could maintain and provide nutrition to spermatozoa or extracellular cryoprotectants. However, cryoprotectants could damage cells because they are toxic, so special protection is needed for cryoprotectants during the crystallization process, which was determined based on the type and concentration of the cryoprotectant (Wahjuningsih et al., 2019). AndroMed® is composed of ingredients needed during the cryopreservation process consisting of sodium and potassium, which are essential for maintaining the integrity of the plasma membrane (Pratiwi et al., 2014). However, the cryopreservation process can damage the plasma membrane due to the formation of lipid peroxides, which can cause peroxidation damage to unsaturated fatty acids in the membrane (Wahjuningsih et al., 2021). After the freezing process, the viability of the spermatozoa at T1 was (67.53 ± 3.34), which was higher than T0 and T2. These results are lower than the research performed by Bintara et al. (2015) at 92 ± 3.33.

Based on Table 2, the abnormality on T1 (2.40 ± 0.79) was higher than that of T2 (2.27 ± 0.49) before freezing. Lipid peroxidation can lead to changes in the structure of spermatozoa, low motility, very fast metabolic processes, and intracellular components (Grötter et al., 2019). The results of the study on abnormal spermatozoa showed no significant difference in all treatment groups ($p > 0.05$). The abnormality of sperm in T1 was lower than in T0 and T2 (Table 1). Figure 1 shows abnormalities in spermatozoa, such as a head that is distinct from the tail and a tiny head. These abnormalities can be categorized as primary abnormalities associated with the head or acrosome, secondary abnormalities that occur when cytoplasmic droplets are present in the midpiece or tail, and tertiary abnormalities that affect the tail (Susilawati, 2011). At the time of measurement after freezing, abnormality on T0 (4.02 ± 1.34) was higher than that of T2 (3.21 ± 0.41). This discrepancy may have occurred because the head and tail of the spermatozoa were cut off during the measurement. The concentration of glycerol used during the freezing process can also affect normal morphology since glycerol serves as an intracellular cryoprotectant that can prevent damage during the freezing process (Öztürk et al., 2020). The addition of glycerol with different percentages affects the percentage of abnormality so the greater the glycerol concentration, the lower the rate of reduction of abnormality (Hikmawan et al., 2016).

The TSM can be determined by multiplying the spermatozoa concentration by the volume of semen (Susilawati et al., 2020). The TSM results showed no significant difference among the treatment groups ($p > 0.05$). The percentage of total spermatozoa motile was the highest in T2 and lowest in T1 (Table 1). The TSM before freezing at T2 was 52.73 ± 0.41 million/straw, which was higher than in T1 (50.00 ± 1.18 million/straw) and T0 (45.00 ± 1.15 million/straw). This difference in TSM is attributed to lipid peroxidation during dilution and preservation (Bucak et al., 2013). Cold storage at a temperature of 4-5°C can result in cold shock and increase lipid peroxidation (Bucak et al., 2012). The higher value of TSM indicates high frozen semen quality (Tethool et al., 2021). However, However, after freezing, TSM decreased in all treatment groups, with T2 having the highest value of 17.25 ± 1.69 million/straw followed by T0 and T1. The decrease in TSM can be attributed to the low TSM value before freezing and the formation of ice crystals that can cause intracellular damage and cell death during the freezing process (Tethool et al., 2022). To prevent ice crystal formation, egg yolk phospholipids, and soy lecithin can be used to form a protective layer on the membrane, but it is important to note that these compounds may not penetrate the membrane (Kakati et al., 2019).

Table 3. Kinematic parameters after freeze-thawing of Saanen goats' semen using different diluents

Kinematic parameters	T0	T1	T2
Motile (%)	53.95 ± 3.88	57.70 ± 3.66	60.00 ± 3.48
Progressive (%)	37.13 ± 3.66	31.93 ± 3.62	40.22 ± 4.70
APL (µm)	24.17 ± 4.12[a]	23.55 ± 4.05[a]	30.44 ± 5.77[b]
DCL (µm)	30.66 ± 8.19	30.20 ± 8.15	34.46 ± 8.67
DSL (µm)	30.91 ± 2.78	30.17 ± 2.79	34.90 ± 4.48
AVP (µm/sec)	67.12 ± 10.28	63.07 ± 12.56	80.20 ± 16.61
VSL (µm/sec)	58.45 ± 8.61	56.93 ± 11.30	73.92 ± 15.58
VCL (µm/sec)	111.82 ± 17.11[b]	101.89 ± 19.67[a]	108.93 ± 21.79[b]
STR (%)	57.23 ± 7.03	66.71 ± 5.33	63.83 ± 7.09
LIN (%)	37.34 ± 4.84[a]	45.76 ± 3.43[b]	48.29 ± 5.42[b]
ALH (µm)	4.45 ± 0.75	3.75 ± 0.71	3.41 ± 0.61
BCF (Hz)	21.41 ± 2.49	24.32 ± 2.51	23.70 ± 3.52
WOB (%)	42.12 ± 5.39	46.85 ± 3.80	48.07 ± 4.19

[ab]Different superscripts with rows indicate significant differences at $p < 0.05$. T: Treatment, T0: Tris-egg yolk, T1: AndroMed® and T2: OviXcell®. APL: Average path length, DCL: Distance curved line, DSL: Distance straight line, AVP: Average velocity path, VSL: Velocity straight line, VCL: Velocity curved line, STR: Straightness, LIN: Linearity, ALH: Amplitude of lateral head, BCF: Beat cross-frequency

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The data in Figure 2 is used as input for the Computer-Assisted Sperm Analysis (CASA) software, specifically the CASA IVOS II by Hamilton Thorne in the USA. The software analyzes various sperm parameters such as straight-line velocity (STR), the amplitude of lateral head displacement (ALH), beat cross frequency (BCF), and curvilinear velocity (WOB). In addition to these factors, the software also tracks various types of sperm motion, such as motile (green), progressive (aqua), slow (pink), static (red), border crosser (blue), and late track (yellow).

Based on the results of the study, the kinematic parameters consisted of 13 parameters using a different diluent. Results showed that sperm motility using OviXcell® diluent (T2) was higher than using tris-egg yolk (T0) and AndroMed® (T1), with no significant difference between treatments. Motility correlates with the ability of the sperm to fertilize the ovum (Wahjuningsih et al., 2021) and is crucial for success in penetrating the oocyte, the lower the sperm motility, the lower the success in penetrating the oocyte (Mocé et al., 2022). The motility of fresh semen used was 70% (Isnaini et al., 2019). The fertilization stages include capacitation of spermatozoa and maturation of spermatozoa, attachment of spermatozoa to the zona pellucida, acrosome reaction, penetration of the zona pellucida, meeting of sperm and oocyte (Yekti et al., 2017). Regarding motility, T2 had a higher percentage than T1 and T0, indicating that motility was affected by the concentration of spermatozoa and the diluent used (Yeste et al., 2018). The highest motility was obtained using OviXcell® diluent. The content in OviXcell® diluent contains soy lecithin so that it can provide nutrition to maintain its motility (Fernandes et al., 2021). This is consistent with the results of research that diluent containing soy lecithin takes longer to maintain spermatozoa motility (Khalifa et al., 2013). Soy lecithin in OviXcell® diluent could replace egg yolk lecithin during freezing with a higher motility percentage of OviXcell® diluent than tris-egg yolk (Akourki et al., 2018). In addition, spermatozoa stored at 4-5°C had greater motility than goats at 17-20°C (Xu et al., 2009). Motility decreases if the concentration and duration of storage are at 5°C (Wahjuningsih et al., 2012).

Motile and progressive are level 1 parameters in kinematic analysis using CASA (Ratnawati et al., 2017). The results of the motile percentage indicated the highest value in T2 (40.22 ± 4.70%), which was not significantly different from T0 (37.3 ± 3.66) and T1 (31.93 ± 3.62, $p > 0.05$). The highest progressive at T0 was due to the higher percentage of motility among the treatments, namely T0 and T1. However, T0 showed higher than T1 due to differences in the time of equilibration in T0 and T1. Progressive detection using CASA in research results was higher than in previous reports which indicated that progressive motility was 3.5 ± 0.4% (Bezerra et al., 2011). These results are lower than the research by Vázquez et al. (2015) at 34.6 ± 21.4. This could be due to the use of different glycerol (only 6%), while in this study it was 13%. The function of glycerol was as an intracellular cryoprotectant to prevent cold shock so that progressive spermatozoa could be protected (Sikarwar and Ramachandran, 2020). It was revealed that the addition of glycerol can cause osmotic damage and is toxic to spermatozoa if the percentage used is not appropriate (Sariözkan et al., 2010).

Based on the APL results obtained, there was a significant difference between the treatments ($p < 0.05$) with the highest yield being 30.44 ± 5.77 in the T2 treatment and the lowest in T1 of 23.55 ± 4.05 µm using the AndroMed® diluent followed by the T0 diluent of 24.17 ± 4.12. Average path length is the average direction of the path taken by the spermatozoa head (Kaewkesa et al., 2016). Significant differences between treatments in APL were caused by different levels of antioxidants resulting from diluent components, cryopreservation time, and freezing procedures (Naijian et al., 2013). The antioxidant content in egg yolks is β-carotene which gives egg yolks a yellow color pigment (Kutluyer et al., 2014). The function of antioxidants is to minimize the oxidation process of fats and oils (Yousr and Howell, 2015). Furthermore, DCL results indicated no significant difference between treatments ($p > 0.05$) with the lowest in T1 at 30.20 ± 8.15, followed by T2 (34.46 ± 8.67) and T0 (30.66 ± 8.19). The DSL results in the T2, T0, and T1 treatments were 34.90 ± 4.48, 30.91 ± 2.78, and 30.17 ± 2.79, respectively. The research results of Maylem et al. (2017) revealed that the results of APL, DCL, and DSL were 54.29 ± 21.48, 96.15 ± 39.87, 45.72 ± 21.6, respectively. The reported values were higher than those presented in a study by Wang et al. (2021).

The results of the AVP study showed that there was no significant difference among all treatment groups ($p > 0.05$). The results for T2 were higher than T0 and T1. The highest VSL results were obtained at T2 and the lowest at T1 and T0. Velocity curved linear showed significant results with differences between treatments, namely T0 was higher than T1 and T2 ($p < 0.05$). Velocity curved linear is an assessment indicator for power in the movement of spermatozoa so that it does not describe the direction of movement but provides complementary information (Susilawati et al., 2018). The results of AVP, VSL, and VCL were 30.35 ± 0.478, 18.50 ± 0.288, and 63.75 ± 0.47 respectively, in post-thawing of tris-egg yolk diluent (Anand and Yadav, 2016). This can lead to the production of reactive oxygen species (ROS), changes in membrane permeability, and the formation of intracellular ice crystals. The presence of ice crystals can result in increased osmotic pressure and alteration of sperm function, leading to changes in dissolved substances and damage to the plasma membrane. These changes can decrease sperm motility and increase damage, ultimately affecting capacitation and acrosome reactions (Wahjuningsih, 2019; Prastiya et al., 2021).

The results of the study showed that there was no significant difference ($p > 0.05$) in STR of spermatozoa between T0, T1, and T2, with STR values of 57.23 ± 7.03, 66.71 ± 5.33 and 63.83 ± 7.09 respectively ($p > 0.05$). The STR is an indicator of moving in a straight line on the average spermatozoa (Ratnawati et al., 2017). The lowest STR value was obtained using tris-egg yolk diluent at 57.23 ± 7.03%, which was lower than the results reported by Monteiro et al.

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(2022) at 85.97 ± 3.26. The use of egg yolk as a cryoprotectant can reduce sperm quality, hence the addition of extracellular cryoprotectants like soy lecithin in OviXcell® and AndroMed® diluents resulted in higher STR values, as reported by Sariözkan et al. (2010). Wang et al. (2021) reported STR values of 76.99 ± 8.67, which were lower than the results of this study.

The LIN of spermatozoa showed a significant difference ($p < 0.05$) between T0, T1, and T2, with the highest value obtained in T2 at 48.29 ± 5.42 and the lowest in T0 at 37.34 ± 4.84 and T1 at 45.76 ± 3.43. Linearity is an indicator of the straightness of the spermatozoa trajectory (Ratnawati et al., 2017). Research by Sadeghi et al. (2020) indicated that LIN value was 59.4 ± 1.2 in 24 hours of storage and 60.5 ± 0.9 in 48 hours of storage. This means that the storage of spermatozoa at 4-5˚C can decrease LIN values but increase motility. Efforts can be made to prevent cold shock by slowing down enzymatic reactions to maintain shelf life by reducing metabolic activity reversibly (Freitas-Ribeiro et al., 2019).

The ALH was highest in T0 at 4.45 ± 0.75, followed by T1 at 3.75 ± 0.71 and T2 at 3.41 ± 0.61 with no significant difference between the groups ($p > 0.05$). The ALH is the average width of the vibration oscillation or vibration of the spermatozoa head when moving (Ratnawati et al., 2017). The highest ALH value obtained in this study was lower than the value reported by Vázquez et al. (2015) at 5.624. Kathiravan et al. (2011) stated that ALH is a parameter that can indicate the average width of the oscillations when the spermatozoa heads swim. The ALH in the current study was <5 µm, which was still relatively stable after freezing.

The results of the BCF study showed that T1 was higher at 24.32 ± 2.51 compared to T2 at 23.70 ± 3.52 and T0 at 21.41 ± 2.49. In each treatment, the results showed no significant difference ($p > 0.05$). The BCF refers to a total of spermatozoa passages at an average rate per second (Ratnawati et al., 2017). The values obtained in this study were higher, compared to the results reported by Arangasamy et al. (2018), which were 10.2 ± 0.3 in goat semen. The values in this study indicate that the average flow per second has a path in a stable condition with a range of values > 20 Hz in each treatment. These results illustrate the strong frequency of spermatozoa movement. This relationship can be connected if the high ALH and low BCF values indicate a low kinetic speed.

The WOB results showed no significant difference among the treatments ($p > 0.05$) with the highest value observed in T2 at 48.07 ± 4.19 followed by T1 at 46.85 ± 3.80 and T0 at 42.12 ± 5.39. The WOB is a measurement of trajectory oscillation with a strength of one second (Ratnawati et al., 2017). The highest results were obtained using OviXcell® diluent and the lowest using tris-egg yolk diluent. The content of soybean lecithin in the diluent provides nutrition to the spermatozoa so that it strengthens the oscillations and kinematic efficiency can be known to be high or low. The results of research by Monteiro et al. (2022) showed a WOB result of 74.92 ± 8.29 using the tris-egg yolk diluent and 72.54 ± 5.04 using the skim milk diluent. Comparable results were obtained in another study at 70.01 ± 0.17 after freezing using a commercial tris-egg yolk diluent (Barbas et al., 2018).

CONCLUSION

In conclusion, the use of various diluents, such as tris-egg yolk, AndroMed®, and OviXcell®, can maintain the quality of frozen spermatozoa for over 24 hours, including motility, viability, abnormality, and TSM. Kinematic parameters obtained using CASA IVOS II can provide relevant information for various parameters using these diluents. The visual motility can be aligned to the ability of speed, distance, average width, and path length of spermatozoa which can be clearly detected. These results can be applied in further research involving direct artificial insemination.

DECLARATIONS

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Authors’ contributions

Lailatun Nisfimawardah, Aulia Firmawati, Muhammad Nur Ihsan, Trinil Susilawati, and Sri Wahjuningsih contributed manuscript writing, data analysis, and study design. Lailatun Nisfimawardah collected data analysis from the field and laboratory. All authors contributed review data from this research, statistical analysis, and approved final draft of the manuscript.

Ethical consideration

The authors declare that this manuscript has been checked by all authors, and it was originally submitted to the journal so that it is publishable for the first time in this journal.

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Competing interests

The authors state that there is no personal interest and financial interest in this research.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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Prevalence of Intestinal Protozoa in Pigs of Northern Black Sea Region, Ukraine

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ABSTRACT

Intestinal protozoan parasites threaten the health and welfare of pigs and impair the sustainability of pig farms, resulting in monetary losses. The present study aimed to determine the distribution of protozoa in large white pigs in the farms of Odesa, Mykolaiv, and Kherson regions in Ukraine. The parasitological surveys were conducted from March 2020 to March 2022 on three types of farms, including four large farms (> 100 sows), six medium farms (25-100 sows), and eight small farms (< 25 sows). A total of 3938 fecal samples from pigs of various age groups, namely 0-2-month piglets, 2-4-month piglets, pigs on fattening, and sows, were examined. *Eimeria* and isospores were determined using McMaster's method in Raynaud's modification, cryptosporidia by staining smears, blastocysts by the method of ethyl acetate-formalin concentration, and direct microscopy. The recorded protozoa were species *Eimeria* spp., *Cystoisospora suis* (syn. *Isospora suis*), *Balantidium coli*, *Cryptosporidium* spp., *Blastocystis* spp. These species of protozoa were observed in 31.1%, 49.0%, and 58.8% of pigs in large, medium-sized, and small farms, respectively. The findings indicated that *Isospora suis* and *Eimeria* spp. were most commonly present in piglets aged 0-2 months in large (29.7% and 23.0%, respectively), medium (32.3%, 29.4%), and small farms (30.0%, 13.5%). *Balantidium coli* was not registered in pigs from large farms, and in piglets 2-4 months old from small farms, the prevalence of infestation was 17.2%. *Cryptosporidium* spp. and *Blastocystis* spp. were mostly recorded in piglets 2-4 months old from small farms (16.2%, 7.1%). In large and medium-sized farms of the Northern Black Sea region, mono infestations were recorded the most (73.6%, 72.0%), while mixed two-component infestation dominated (52.5%) small farms. Intestinal protozoa should be considered in the differential diagnosis of intestinal disorders as major factors or concomitant intestinal pathogens.

Keywords: Age, Animals, Black Sea, Intestinal protozoa, Prevalence

INTRODUCTION

Intestinal parasites have a significant negative impact on the efficiency of pig farming. Young animals are particularly vulnerable to parasitic infections as they can cause diarrhea and dehydration, and consequently the death of the animals. Parasites can also hinder weight gain in pigs, causing economic losses (Li et al., 2020). The economic effectiveness of combating parasitosis relies on the ability to minimize or eliminate losses and manage the costs associated with disease prevention and treatment. A crucial requirement for accurate profit and loss assessment is determining the ultimate loss resulting from parasitic infections in farm animals. This information is essential for precisely evaluating the financial impact and making informed decisions regarding disease control strategies (Michalski, 2007).

Internal parasites are highly prevalent in pigs, making it essential for every producer to be aware of their presence and the associated losses they can cause. The amount of loss is affected by several factors, the most important of which are the species of endoparasites, housing, feeding, geographical location, and breed of animals (Roepstorff et al., 2011; Zakir Abadura et al., 2022). At the beginning of the infestation, the animal loses its productive potential due to the inability to adapt to endoparasites. However, as the animals grow, they undergo physiological adjustments and their immune system develops, enabling them to better modulate any damage caused by parasites and compensate for the increase in weight (Weng et al., 2005; Lotfalizadeh et al., 2022). Protozoa are major biological barriers to efficient pig production but are often overlooked because clinical symptoms are rarely detected. Infected pigs experience a 5% reduction in daily feed intake and a 31% average daily growth, as well as an average 17% higher feed conversion ratio, compared to pigs on a parasite-free diet (Kipper et al., 2011; Ózsvári, 2018).

Gastrointestinal parasites are a major cause of reduced production efficiency in pigs. They impact productivity by directly competing for nutrients needed for optimal growth and reproduction. Additionally, these parasites can cause tissue damage (lesions) leading to organ culling in meat inspection, poor feed conversion, diarrhea, dehydration, or even

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death of animals (Kochanowski et al., 2017). The prevalence of gastrointestinal parasite infections in pigs varies based on different factors, including housing systems (intensive and semi-intensive), deworming practices, and pig management (Eijck and Borgsteede, 2005; Weng et al., 2005; Nwafor et al., 2019; Symeonidou et al., 2020; Adhikari et al., 2021). Free-range pig farming is common in rural areas of many developing countries despite some disadvantages, such as poor feed conversion, high mortality, and poor production (Kagira et al., 2010).

In most cases, gastrointestinal parasitic infections in pigs are subclinical, meaning they do not show noticeable symptoms. However, symptomatic infections can occur, particularly in young pigs. The most common mistakes of pig owners in the fight against parasitic infections are the lack of periodical examination of animal feces to identify specific parasitic problems on the farm, incorrect administration of antiparasitic drugs, and ineffective disinfection of premises (Balicka-Ramisz et al., 2020; Li et al., 2022; Sadr et al., 2022).

Pig endoparasitism indicates heterogeneity in terms of the involved parasite species and their pathogenicity (Roepstorff et al., 1998; Schubnella et al., 2016). Additionally, parasitized pigs tend to be more susceptible to infectious and non-infectious diseases, which undermine their health and welfare status (Greve, 2012). Acquisition of parasite-free pigs combined with good hygiene practices can minimize the initial infection pressure and further infection of the herd to a minimum (Joachim et al., 2001). The impact of endoparasites depends on the parasite load and the individual resistance of the animal, which can be influenced by environmental and nutritional factors. Endoparasitism can occur with or without clinical symptoms. A disease with a clinical manifestation can lead to death, especially in the initial phase of growth. The absence of clinical symptoms is important for production as if it remains unnoticed, it can lead to economic losses due to reduced pig productivity (Delsart et al., 2022).

The aim of the current research was to determine the prevalence of intestinal protozoa in pigs of different age groups depending managing system in the Northern Black Sea Region, Ukraine.

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Scientific Council of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" of the National Academy of Agrarian Sciences of Ukraine. The experiments performed on animals do not contradict the current legislation of Ukraine (Article 26 of the Law of Ukraine 5456-VI of 16.10.2012 "On protection of animals from cruel treatment") and "General ethical principles of animal experiments", adopted by the First National Congress of Bioethics and international bioethical standards (materials of the IV European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes, Simmonds, 2018; Kabene & Baadel, 2019). The research program was reviewed and approved by the Bioethics Commission of the National Research Center "Institute of Experimental and Clinical Veterinary Medicine" in the current order.

Sampling time and study design

From March 2020 to March 2022, a total of 3938 samples of feces from pigs of different age groups were examined. The samples were collected from Odesa, Mykolaiv, and Kherson regions. The main criteria for the selection of fecal samples were lack of appetite, presence of diarrhea, and inactivity. Animals in these farms were not subjected to any treatment or administration of antiprotozoal drugs. In the parasitology laboratory, 1267 fecal samples from pigs with different age groups from large farms were examined (> 100 sows), namely Bolgradskyi district (n = 320), Podilskyi (n = 295), Berezneguvatskyi (n = 402), Skadovsky (n = 250). Of the total fecal samples, 1774 were from medium farms (25-100 sows), namely Kodymskyi (n = 300), Baltskyi (n = 210), Tatarbunarskyi (n = 255), Veselinivskyi (n = 372), Arbuzynskyi (n = 306), and Ivanivskyi (n = 331) and 897 samples were from small farms (<25 sows) in 8 districts of the Northern Black Sea Region of Ukraine (Figure 1).

Figure 1. The studied farms of the Northern Black Sea region of Ukraine from 2020 to 2022.
Large (> 100 sows); Medium (25–100 sows); Small (<25 sows)

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Fecal sampling and parasitological analysis

Fecal samples from piglets aged 0-2 months old (n = 1245) were collected directly from the rectum. In each litter, samples were obtained from 3 to 5 piglets and combined into a single pooled sample. In addition, fattening pigs and sows were sampled directly from the rectum or from the floor immediately after defecation. Feces were analyzed using McMaster's method in Raynaud's modification, and the number of oocysts per gram (OPG) of fecal value was estimated according to the method of Raynaud (1970). Samples were examined at 100x magnification and in doubtful cases at 400x magnification. In order to determine cryptosporidia for coprological studies, 2 fecal samples from each animal were prepared on clean and degreased glass slides. Each sample was examined by creating a native smear according to the generally accepted method. The smears were stained according to the Kester and Romanovsky-Giems method, followed by microscopy at a magnification of x50. To isolate oocysts, a similar technique to the one described by Maddox-Hyttel et al. (2006). Blastocysts detection was carried out using the method of ethyl acetate-formalin concentration and direct microscopy (Vielma, 2019).

Statistical analysis

The chi-square test with Yates correction was used to compare the prevalence of intestinal protozoa in piglets and sows of different types of farms. The OPG values obtained in piglets and sows in different types of farms were compared using the Kruskal-Wallis test (in exceptional cases, the Kolmogorov-Smirnov test was used for the analysis of *I. suis* OPG in sows). Differences were considered statistically significant at $p < 0.05$. All data were analyzed using STATISTICA 7.1 (StatSoft).

RESULTS

In large farms (>100 sows), protozoa were detected in 31.1% of pigs from different age groups. In piglets aged 0-2 months old, the total infestation was 41.9%. Of all detected protozoa, *Isospora suis* was recorded at the highest rate (29.7%), while *Balantidium suis* (*B. suis*) was not detected (Table 1). The total infestation in piglets aged 2-4 months old was 38.9% and the most common was *Eimeria* spp. (21.3%), while the infestation of *Isospora suis* decreased by 17.4%. In fattening pigs (6-10 months) and sows, the total rates of infestation were 12.5% and 22.9%, respectively, with the dominance of *Eimeria* spp. 8.0% and 8.1%. In medium-sized farms (25–100 sows), out of 1774 examined animals of different age groups, the incidence of protozoa was 49.0%, which was 17.9% more than the incidence of pigs in large farms (Table 2).

Piglets aged 0-2 months old were most affected by oocysts of *Isospora suis* (32.3%) and *Cryptosporidium* spp. (13.1%), the total percentage of infestation was 59.4. The total infestation of piglets aged 2-4 months old amounted to 65.4% with the dominance of *Eimeria* spp. (29.4%) and *Cryptosporidium* spp. (10.3%). Infestation of fattening pigs with protozoa was 26.0%, and the prevalence rate of *Eimeria* spp. was 8.6%. The total infestation in sows was 23.7% and was dominated by *Blastocystis* sp (5.0%). Out of 897 examined pigs of different age groups in small farms (<25 sows), 58.8% were affected by protozoa (Table 3).

Piglets within the age range of 2-4 months (74.9%) and 0-2 months (67.1%) were the most infected groups of age, respectively. In fattening pigs and sows, the infestation percentage by protozoa was almost at the same level and amounted to 31.6% and 35.7%, respectively. Piglets aged 2-4 months old were most affected by *B.* suis (17.2%) and *Cryptosporidium* spp. (16.2%), compared to animals with large and medium farms. Pigs of all ages in large farms were dominated by mono infestation with protozoa, which ranged 57.4-73.6% (Graph 1). Double infestation was recorded in 22.7-32.1% of pigs, while the maximum rates of triple infestation (14.9%) were recorded in piglets of 2-4 months.

Single-component infestation in pigs from medium-sized farms was recorded at almost the same level as in pigs from large farms (Graph 2). However, the two-component infestation was recorded in a larger number of pigs with an incidence rate of 24.8-30.4%. The prevalence of protozoa caused by three pathogens was high only in piglets aged 2-4 months old (9.6%). In contrast to large and medium-sized farms, two-component infestation, as well as one-component infestation, was dominant in small farms, which was the highest in piglets aged 2–4 months old (52.5%, Graph 3). In sows, the extent of damage by triple infestation was recorded in 20% of animals.

Table 1. The prevalence of protozoa in pigs of large farms (>100 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022.

Age groups	Animals (number)	The extent of the infestation (%)				
		Isospora suis	***Eimeria* spp.**	***Balantidium suis***	***Cryptosporidium* spp.**	***Blastocystis* spp.**
0–2 months	415	29.7	23.2	–	6.9	1.9
2–4 months	380	12.3	21.3	–	3.1	2.1
on fattening	350	1.7	8.0	–	1.1	1.7
Sows	122	5.7	8.1	–	4.9	4.1

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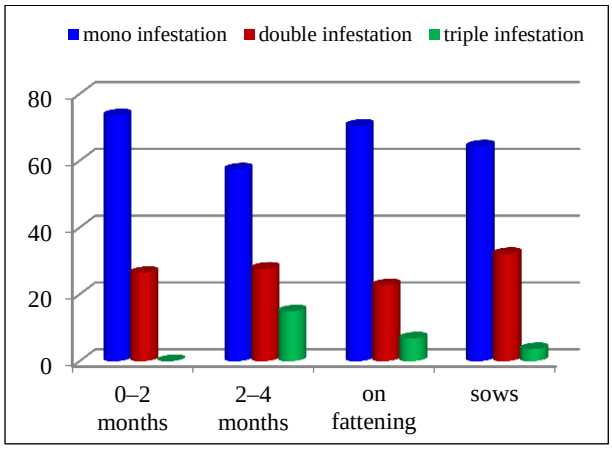
Table 2. The prevalence of protozoa in pigs of medium farms (25-100 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022

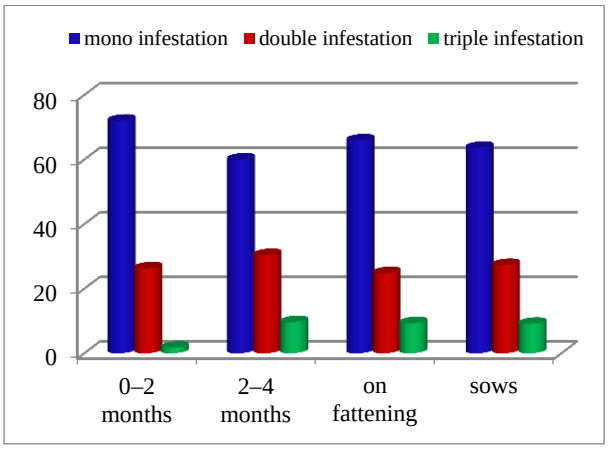
Age groups	Examined animals (Number)	The extent of the infestation (%)				
		Isospora suis	*Eimeria* spp.	*Balantidium suis*	*Cryptosporidium* spp.	*Blastocystis* spp.
0–2 months	510	32.3	4.3	5.7	13.1	3.3
2–4 months	630	14.4	29.4	6.5	10.3	4.1
on fattening	495	4.2	12.3	5.2	0.4	3.8
Sows	139	4.3	8.6	2.1	3.6	5.0

Table 3. The prevalence of protozoa in pigs of small farms (< 25 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022

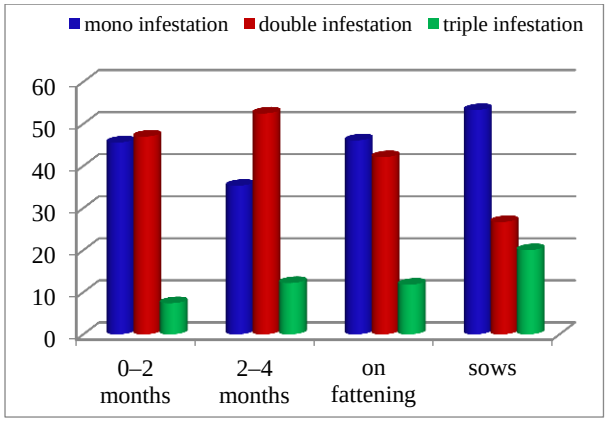
Age groups	Examined animals (Number)	The extent of the infestation (%)				
		Isospora suis	*Eimeria* spp.	*Balantidium suis*	*Cryptosporidium* spp.	*Blastocystis* spp.
0–2 months	320	30.0	7.5	11.2	12.8	5.6
2–4 months	295	20.6	13.5	17.2	16.2	7.1
on fattening	240	4.5	7.9	12.9	2.5	3.8
sows	42	2.3	11.9	9.5	4.7	7.1



Graph 1. Distribution of mono- and mixed protozoa in pigs of large farms (>100 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022



Graph 2. Distribution of mono- and mixed protozoa in pigs of medium farms (25–100 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022



Graph 3. Distribution of mono- and mixed protozoa in pigs of small farms (<25 sows) located in the Northern Black Sea Region of Ukraine from 2020 to 2022

DISCUSSION

Parasitism is an example of an antagonistic trophic relationship, where an organism exploits another species (known as the host) as a habitat and source of nutrients, either temporarily or permanently. It negatively affects the host's condition, and can cause the host's death (Vannier-Santos and Lenzi, 2011).

The effects of parasites on the body of the host depend on various factors, including the intensity of the infection, the age of the host, the host's immune response, its living conditions, feeding practices, and other environmental factors.

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All over the world, losses caused by parasitic diseases in animal husbandry are enormous (Symeonidou et al., 2020).

According to a study by Schubnell et al. (2016), parasite infections in pigs during the first months of life are highly prevalent. While helminths were uncommon, protozoan parasites were frequently detected and *I. suis* appears to be the most important parasite species associated with diarrhea or emaciation in the studied pig population.

On the territory of the Poltava region (Ukraine), eimeriosis and isosporosis were widespread protozoans with average infestation rates of 49.8% and 8.3%, respectively. Coccidiosis occurs more often as part of mixed invasions of the alimentary tract of pigs (73.2%) together with nematodes of the *Oesophagostomum genus*, *Ascaris suum*, *Trichuris suis* and the simplest of the *B. suis*. Eimeriosis and isosporosis mono invasions were recorded less. *Balantidium suis* (EI - 19.5%) are the main comembers of *Eimeria* and *isospores* in the intestines of pigs (Yevstafieva and Kovalenko, 2019).

The incidence of isosporosis and eimeriosis were significantly higher in young piglets, compared to adults. This implied that juveniles were highly susceptible to coccidia because of their weaker immunological response to severe infections. Several species of *Eimeria* and *Cystoisospora* could infect pigs, with *Cystoisospora suis* (syn. *Isospora suis*) being the predominant coccidia (Mundt et al., 2005; Worliczek et al., 2007). This apicomplexan protozoan mainly affects suckling piglets, which are unable to mount an adequate primary immune response (Koudela and Kucerová, 1999).

In a study performed by Symeonidou et al. (2020), approximately 19.1% of suckler piglets and 9.1% of weaned piglets were infested, while only four cases of *C. suis* infection were detected in sows. The prevalence of *Cryptosporidium suis* (*C. suis*) infections in pig populations varies depending on age and geographic location. In studies conducted in Europe, it has been observed that an increase in immunity against *C. suis* depended on age. The *C. suis* infections were more commonly found in suckling piglets, with *Isospora suis* being the predominant parasite in piglets aged 2-3 weeks, accounting for 26.9% of cases. *Cryptosporidium parvum*, another species of Cryptosporidium, was detected in 1.4% of the investigated piglets (Wieler et al., 2001).

Cryptosporidium suis in suckling piglets ranged from < 1% to > 40% (10.0% in Germany (Damriyasa and Bauer, 2006), 12.8% in Switzerland (Schubnell et al., 2016), 20.9% in Southern Germany (Wieler et al., 2001), and 42.9% in Poland (Kochanowski et al., 2017). For Scandinavian countries, *C. suis* infestation rate was 19.5% in Denmark, 4.5% in Finland, 31.8% in Iceland, 0.3% in Norway, and 20.1% in Sweden (Roepstorff et al., 1998).

The prevalence of intestinal parasites was investigated in intensive pig farms in Guangdong Province, China. The findings indicated that 24.9% of pigs were affected by coccidiosis (*Eimeria* spp. and *Isospora suis*) and 47.2% by *Balantidium coli* (*B. coli*). These infected pigs were mainly from farms without a strategic antiparasitic treatment regime (Weng et al., 2005). In some cases, *B. coli* was thought to be a major factor in porcine colitis although this role has not been fully elucidated (Szczotka-Bochniarz et al., 2021).

Giarratana et al. (2021) reported *Balantidium coli* in 46.89% of pigs, with a significantly higher prevalence in commercial hybrid pigs (64.84%) than in local breeding pigs (27.91%). The infection was more common in pigs raised in an intensive breeding system than in an extensive one. The prevalence of infection was lower in older animals than in young ones. The breeding system is likely to be a major factor in the distribution of parasites, as well as the sanitary and hygienic status of farms. Regarding the risk factors for *B. coli* parasitism, they increase with age. Prevalence increased significantly from 57% in suckler piglets to 100% in pigs older than one month (Hindsbo et al., 2000).

Cryptosporidum was confirmed in 27.7% of pig fecal samples. Most of the infected animals (42.1%) were 2 to 3 months old. The following types of parasites were identified, *Cryptosporidum scrofarum*, *C. suis*, and *C. parvum*. Asymptomatic infestations caused by *C. scrofarum* were observed in most herds. Mixed diseases caused by *C. suis* and *C. scrofarum* were rare, however, they were observed in 86% of positive animals (Rzeżutka et al., 2014).

In a study of intestinal parasites on intensive and extensive pig farms in Chongqing, China, *Eimeria spp*. was recorded in 16.53% of pigs, *Isopora suis* in 5.02%, *B. coli* in 22.79%, and *Cryptosporidium* spp. in 6.60%. *Balantidium coli* was the most common protozoan in all age groups of pigs. Season of the year, age of the animals, and treatment methods can affect infection rates (Lai et al., 2011).

Of 402 pigs from 55 farms, *B. coli* cysts, *Cryptosporidium* oocysts, and mixed infection were detected in 51.5%, 13.9%, and 7.2% of pigs, respectively. Among the pigs, the highest infection rates were observed in young pigs. Specifically, 58.9% of the young pigs were found to be infected with *Cryptosporidium* oocysts, 54.6% with *B. coli* cysts, and 58.6% with mixed infections (Yatswako et al., 2007). Parasite prevalence was 93.1% on family farms and 59.1% on industrial farms. Single infections were more frequent (32.5%) than multiple infections (12.1%). In both family and industrial farms, the most common parasites were *B. coli*, with prevalence rates of 71.6% and 46.4%, respectively. The *coccidia* parasites were also prevalent with a prevalence of 71.6% in family farms and 19.2% in industrial farms (Barbosa et al., 2015).

According to Kochanowski et al. (2017), single infestations are common in large and medium-sized farms (57.4-73.6%), while double infestation (26.7-47.0%) and triple infestation (7.4–20.0%) in small farms. A higher prevalence of parasites was found in small farms than in medium and large farms, except for the prevalence of *coccidia*, which was the highest in medium farms. Infection with several pathogens was recorded more often than with one parasite.

In domestic pigs, the diet and rearing conditions change dramatically during the life of the animal, which can cause a difference in the prevalence of mixed infections among different age groups. Masuda et al (2022) found that the

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prevalence of single infections caused by *Blastocystis suis* was 37.8%, while the prevalence of mixed infections was 57.3%. A high percentage of single infections (86.7%) was observed in sows, piglets, and weanlings, while mixed infections (83.3%) were observed in 3-5 month-old piglets and producer pigs.

Dashti et al. (2022) reported that *Blastocystis* spp. was significantly less common in intensively reared Iberian pigs (22.9%) than in their extensively reared counterparts (51.0%) or in intensively reared large white pigs (64.1%). Large white pigs indicated a significantly higher prevalence of *Giardia duodenalis*, *Cryptosporidium* spp., and *Eimeria bieneusi*. *Balantidium coli* was equally common (40.0-51.1%) in all three investigated pig populations.

The results of this study confirmed the difficulty in eliminating intestinal parasitism in pigs, even with regular and systematic antiparasitic prophylaxis. Several factors contribute to this challenge. Firstly, breeding animals, such as lactating sows, have a longer lifespan and can act as reservoirs for parasites, leading to re-infection of other age groups. Secondly, there may be shortcomings in generally accepted treatment schemes in intensive pig farming these schemes often involve the use of preventive substances in feed, regardless of the difference in body weight of sows between age groups, which may cause insufficient dosing. Finally, preventive schemes can be ineffective due to long time intervals (for example, more than 6 months) between the moments of introducing feed to sows, which can create an unprotected time window in the life of breeding stock (Weng et al., 2005; Barbosa et al., 2015).

Many factors can influence the prevalence and intensity of parasitic infection. Factors, such as herd size, floor type, use of an all-in/all-out system, or pen use related to the breeding system, are particularly important (Mejer and Roepstorff, 2006). Therefore, information about the factors affecting various production systems is important for farm development.

CONCLUSION

All over the world, on farms with different capacities, the prevalence of protozoa in pigs was a very serious problem that reduces the productivity of animals. The extent and intensity of parasitic diseases in pigs should be reduced by educating farmers and private animal owners on the value of intensive animal care, environmental sanitation, strategic deworming of pigs using effective broad-spectrum anthelmintics, biological control of parasites, and breaking their life cycle. Further research will be needed on the influence of climatic conditions in different zones of Ukraine on the spread of pig protozoa.

DECLARATIONS

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available.

Funding

None.

Authors' contributions

Mykola Bogach and Ihor Panikar participated in the data collection, analysis, preparation, and revision of the manuscript. Anatoliy Antipov та Volodymyr Goncharenko was involved in the collection of data and laboratory analysis**,** while Olena Bogach formatted and edited the manuscript. All authors read and approved the final manuscripts.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

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Phylogenetic Analysis and Detection of Drug Resistance Gene in *Theileria annulata* Isolated from Buffaloes

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ABSTRACT

Bovine theileriosis, caused by *Theileria annulate*, is disease affecting cattle and buffaloes worldwide. The current study aimed to screen the blood samples of 30 naturally suspected local buffaloes infected with *Theileria* species. The blood samples were initially examined by light microscopic and then the positive samples were subjected to PCR reactions. All 30 animals indicated clinical symptoms, such as high fever, loss of appetite, the presence of the hard tick, and enlargement of lymph nodes. The amplified products of *18S rRNA* were analyzed, along with molecular detection of the drug-binding site alterations and interrelated changes in the *cytochrome b* (*cyto b*) gene. Blood smears revealed the presence of infected erythrocytes with *Theileria* spp. The PCR results confirmed infection in samples when DNA amplified with partial *18S rRNA* and *cyto b* genes. The sequencing data were obtained from GeneBank using the accession numbers OM937770.1, ON207523.1, ON207525.1, ON207524.1, ON207526.1, and ON207527.1 Following BLAST analysis (Basic Local Alignment Search Tool), genetic differences were observed between the Iraqi isolate OM937770.1 and strains from India, Iran, and Turkey. The data obtained from the current study may reveal the genetic alteration of the local strain in the drug-target codons, which are found in one isolate and are different from the GenBank isolates. The results suggest that the failure of buparvaquone therapy might be due to the resistance to *cyto b* gene.

Keywords: Buffalo, Buparvaquone, Gene, *Theileria annulata*

INTRODUCTION

Bovine theileriosis is a worldwide prevalent disease in cattle and buffalo, caused by the tick-borne hemoprotozoan parasite known as *Theileria annulate* (Bilgic et al., 2010; Abdullah and Ali, 2021; Ullah et al., 2021). Several genera of hard ticks (Ixodidae) can transmit the disease and the clinical manifestations include fever, swollen lymphoid tissue, jaundice, and high mortality (Ali et al., 2013; Abdel Rahman and Ismaiel, 2018). Theileriosis negatively affect dairy and livestock animals productivity, leading to significant losses in the industry due to decreased milk output and weight loss (Gharbi et al., 2006). Compared to other vector-borne diseases, theileriosis prevalence is higher than *Anaplasma* spp. (11%) but lower than babesiosis (29%) across the world (Paramanandham et al., 2019; Jacob et al., 2020; Abid et al., 2021). In buffaloes, the infection rates of babesiosis, theileriosis, and anaplasmosis were 51.44%, 15.74%, and 13.99 %, respectively (Anwar, 2018). This discrepancy may be attributed to the fact that most studies have focused on diagnosing theileriosis in cattle rather than buffalos, with cattle traditionally considered the primary host for theileriosis (AL-Judi, 2001; Sallemi et al., 2018; Kawan, 2019).

Apart from the difficulty of species identification, the blood smear technique is not suitable for detecting infections with low parasite levels (Nayel et al., 2012; Rafiullah et al.,2019; Arwa and Kawan, 2022). Serological approaches for detecting *Theileria* species are insensitive due to cross-reactions and the loss of antibodies in long-term carriers (Passos et al., 1998). Therefore, the present study aimed to evaluate the prevalence of *Theileria annulata* (*T. annulata*) among buffaloes in Iraq and identify drug-binding site alterations in codons in the resistant isolates.

MATERIALS AND METHODS

Ethical approval

The project received approval and funding from the local committee of animal care at the College of Veterinary Medicine, University of Baghdad, Iraq, under reference number 706, dated 23/3/2022.

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Sample collection

The study included blood samples of 30 Bubalus bubalis buffalos (28 female and 2 male) aged 1-3 years old, presenting symptoms, such as fever and enlargement of lymph nodes. During the examination of the animals' bodies, the presence of the hard tick species *Haylomma anatolicum* was observed. Subsequently, the animals were treated with buparvaquone (Buparvon, ALKE, Istanbul®), an approved anti-parasitic drug, at a single dose of 2.5 mg/(Saruhan and Paşa, 2008). The treatment was administered in a licensed private veterinary clinic located in southeast Iraq during the early summer of 2021. After the treatment, 5 ml of blood was collected from the jugular vein of each animal using an EDTA-coated glass tube. The blood samples were then transferred to the laboratory of the Zoonotic Diseases Research Unit, University of Al-Qadissyia College of Veterinary Medicine, Iraq, with an icebox.

Microscopic examination

Thin blood films were prepared immediately from the collected blood samples, and subsequently dried, and fixed by 100% Ethanol (BDH, England). Samples were transferred to the laboratory in a slide box for Giemsa staining and examination under a light microscope (Olympus CX21, Philippines) based on the technique described by Soulsby (1982). The slides were stained with Giemsa solution (England), for at least 20 minutes. Finally, the slides were examined using a microscope with an oil immersion lens at a magnification of X100.

Genomic materials extraction

The DNA extraction process was performed on the 30 collected blood samples. For this purpose, 200 µl of whole blood was utilized, and the extraction was conducted using the G-spin genomic kit (iNt RON Biotech. Seongnam, Si, S. Korea) following the manufacturer's instructions. The DNA was successfully isolated from the samples, resulting in concentrations ranging 150-350 ng/µl. Due to issues related to purity and DNA concentration, only 13 samples were used in the subsequent analyses. The concentration and purity of genomic DNA were assessed using a NanoDrop (Thermo/USA), with acceptable 260/230 ratios used for PCR amplification.

Polymerase chain reaction

The primers used in the study were designed according to a corresponding reference sequence, available in the GenBank database of *T. annulata*, 18S rRNA (570bp) gene (OQ411265 and MN432518 (partial) Unpublished data). The forward primer sequence used was F: CAGGCTTTCG CCTTGAATAG, while the reverse primer sequence was R: ACCACCACCCAAAGAATCAA. For the *cytochrome b* (*cyto b*) gene (891 bp), the forward primer employed was F: CGGTTGGTTTGTTCGTCTTT and reverse primer was R: CGAACTCTTGCAGAGTCCAAT (supplied from Bioneer [Korea] in conjunction with the AddStart Taq Master [2x Conc.] 1.0 ml kit/ ADDBIO, INC). The PCR reaction mixture was prepared with a total volume of 20 µl. Each primer (1.5 µl) was added, along with 10 µl of the master mix. A volume of 2 µl of DNA was included, and the remaining volume was filled with deionized PCR water to reach the total volume. The thermocycler reaction protocol included desaturation (at 95°C for 30 seconds), annealing (at 58°C for 30 seconds), extension (at 72°C 1 minute, and final extension at 72°C 5 minutes) for 33 cycles. The amplified products were electrophoresed with 1.5% agarose gel at 80 volts, stained in the ethidium bromide, and visualized with a UV transilluminator reader.

DNA sequencing method

All PCR products were subjected to Sanger dideoxy sequencing technology, following the method described by Hsiao (2019). The amplification of *18S rRNA* gene, and *cyto b* gene was performed, and sequencing was done using the Sanger sequencing system (forward and reverse reaction for each products, Bioneer Company, Korea). The resulting sequence was aligned together for phylogenetic tree analysis using Unweighted Pair Group Method Arithmetic. (UPGM; Wheeler and Kececioglu, 2007). For the tree-building procedure, NCBI-BLAST alignment and Neighbor Distances in MEGA software were used. All data obtained in the current research were submitted to GenBank ON207523.1, ON207524.1, ON207525.1, ON207526.1ON207527.1, and OM937770.1.

RESULTS

The microscopic characterization of the stained blood film indicated erythrocytes infected with the *Theileria* species in only 6 positive samples out of 13 running reactions (Figure 1). These positive samples were obtained from animals exhibiting clinical symptoms, such as high fever, loss of appetite, enlargement of lymph nodes, and pale mucus membranes.

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PCR, sequencing and phylogenetic tree construction

Due to certain limitations, only six out of thirteen PCR-amplified reactions for both the *rRNA* gene, and *cyto b gen* were selected for Sanger dideoxy sequencing technology (Hsiao, 2019, Figure 2). The obtained partial *18S rDNA* sequences were aligned with the corresponding sequences from the GenBank® database (Figure 3). However, only six readable and clean reaction data were obtained from the sequencing process.

The study involved aligning the obtained sequences with corresponding data available in the GenBank database. The alignment was performed for the deduced *cyto b* amino acid sequences, using the Clustal W default setting of MEGA v6.0 (Figure 4). Evolutiocnary distances were calculated using the Unweighted Pair Group MethodArithmetic (UPGMA) method. The phylogenetic tree constructed based on the *cyto b* gene showed distinct distances between local between *T. annulata* isolates and Indian, Iranian, and Turkish strains.

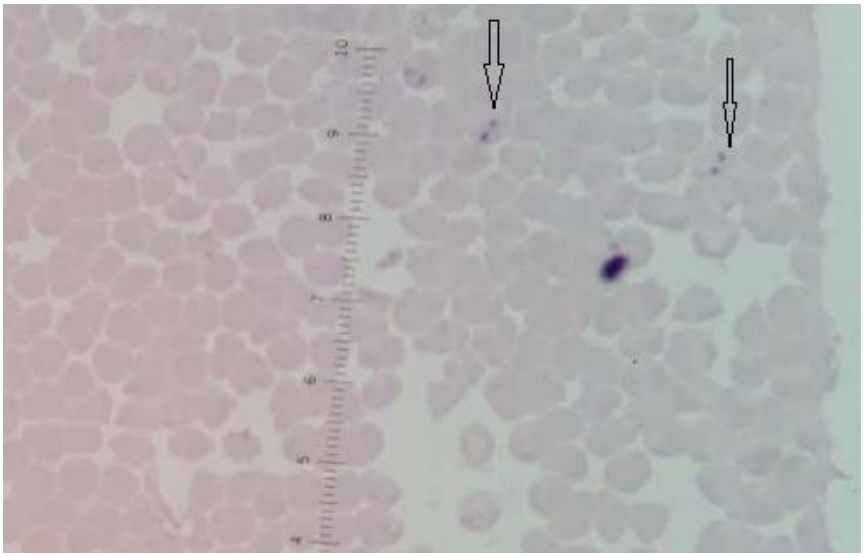

Figure 1. Microscopic examination of stained blood sample by Giemsa staining indicating *Theileria* species (arrows) is in the erythrocytes of a buffalo in Iraq (100 X magnification)

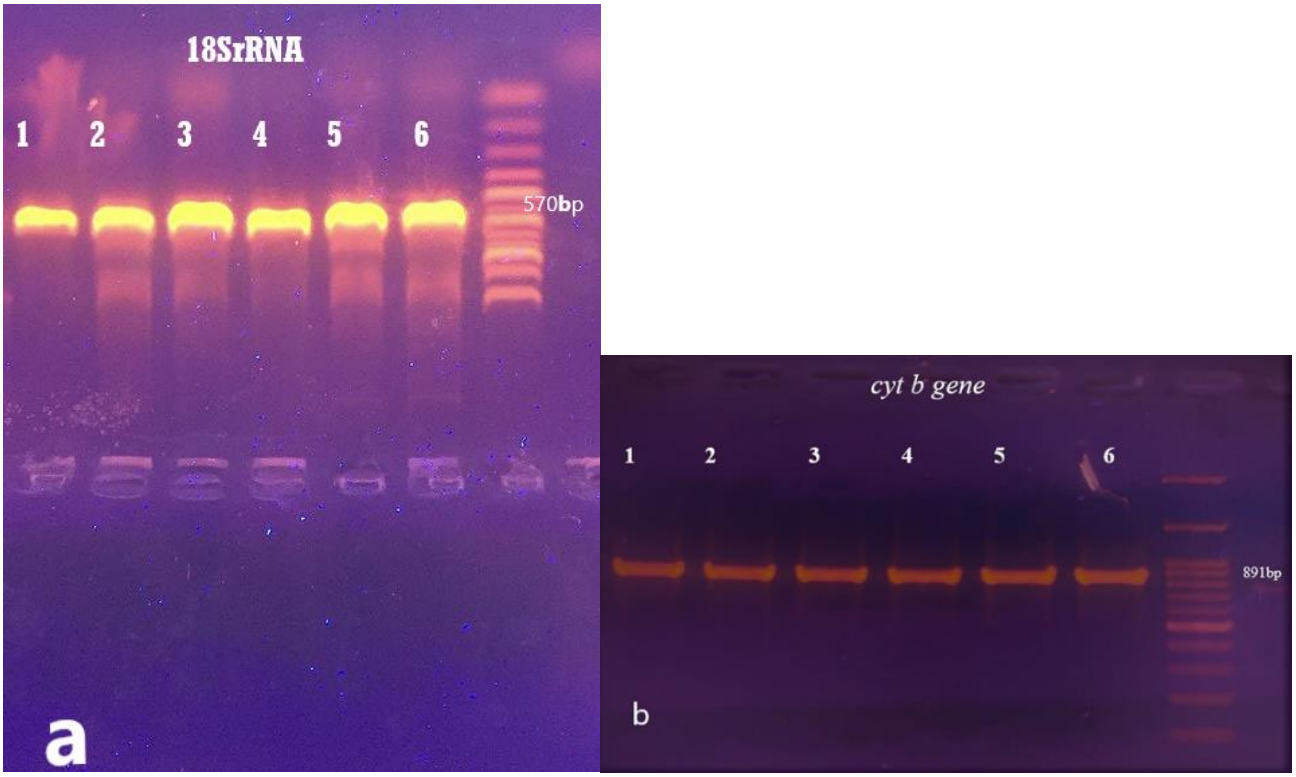

Figure 2. The electrophoresed 1.5% agarose gel using the *Theileria annulata* 18S rRNA and cyto b genes PCR products from buffalo DNA. **a:** Lanes 1-6 represent positive samples at 570bp for 18S (rRNA) gene. **b:** Lanes 1-6 represent positive samples at 891 bp for cyt b gene in buffalo.

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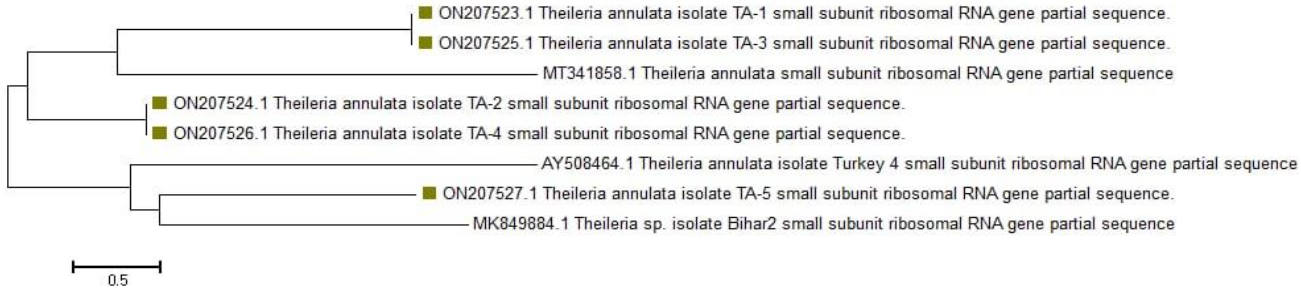

Figure 3. Constricted genetic tree shows Iraqi *Theileria* isolates based on the 18S ribosomal (r.) RNA gene (Green Square). Evolutionary distances were calculated using the (UPGMA) Unweighted Pair Group Method Arithmetic, Wheeler and Kececioglu 2007) method in MEGA v6 in buffalo.

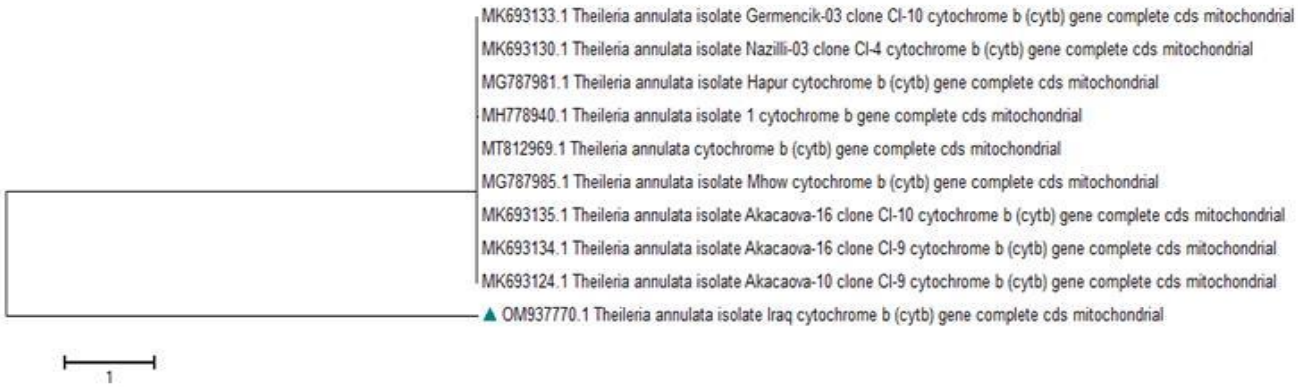

Figure 4. Phylogenetic tree analysis of local *Theileria annulata* isolates based on the (cyto b) gene (green label). The evolutionary distances were calculated using the UPGMA method in MEGA v6.0 (Kumar et al., 2016) in buffalo.

DISCUSSION

Since PCR is a more sensitive method, the results from this investigation were consistent with previous studies (Hasso and Al-Nashy, 2002; Gharbi et al., 2006; Alhaboubi et al., 2017). False-negative diagnoses of theileriosis through blood smear examination often occur due to the various structural configurations of piroplasms (Edith et al., 2018; Farooq et al., 2019; Al-Amery and Al-Amery, 2022). PCR-based molecular diagnosis may be used to circumvent the drawbacks of blood smear testing and investigate the parasite's prevalence throughout a large herd of cattle (Hasso and Al-Nashy, 2002; Faraj 2019; Al-Abedi and Al-Amery, 2021). The tree analysis was conducted using a neighbor-joint algorithm. All the Iraqi obtained sequences of the 18S rDNA aligned together with 100% identity. The isolates ON207523.1 ON207525.1 were close to each other with 100% identity for Indian buffalo isolates with variation with ON207524.1 and ON207526.1 which were close to each other in 100% identity. On the other hand, ON207527.1 was closely attached to Turkish cattle isolates and Indian Buffalo isolates. This result ensures the stability of the *18S rRNA* gene of *T. annulata* in different hosts and the cross-relation of transmission factors and similarity of the buffaloes' origin in Iraq.

Previous studies have indicated that buparvaquone, like other hydroxynaphthoquinones, could well act by binding to the *cyto b* location and preventing the parasite's electron transport pathway (Goodman et al., 2017). The *T. annulata* may become quite drug-resistant, especially in endemic areas, such as Iran, Turkey, and Tunisia, which might make it very difficult for bovine livestock to thrive in these areas (Mhadhbi et al., 2015). The identified drug is a Qo inhibitor that specifically targets the coenzyme Q binding pocket in the c*yto b* gene, leading to effective inhibition of mitochondrial respiration. However, it commonly fails to treat patients with single or double mutations in the Qo binding region of the *cyto b* gene. This finding helps explain the observed genetic distance between the resistant Iraqi isolate (OM937770.1) and strains from India, Iran, and Turkey (Figure 4, Parveen et al., 2021). This is further supported by a study performed in Iraq suggesting several mutations in the *cyto b* gene may have been responsible for the resistance of the parasite against buparvaquone (Alfatlawi et al., 2021).

Theileriosis is of high susceptibility in exotic breeds and crossbred bovines, including buffalo, and its significant effect on animal health leads to economic losses (Al-Taiy et al., 2020). There is no doubt that the treatment with long-standing protocols, may develop drug resistance, especially in the exotic breeds of the infected animals.

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CONCLUSION

Molecular approaches have shown higher specificity in detecting the prevalence of theileriosis compared to blood smear examinations. In this study, it was found that buparvaquone, the primary hydroxynaphthoquinone drug, has become ineffective against tropical theileriosis due to the emergence of drug resistance. The *Cytochrome b* gene plays a crucial role as a target gene and marker in characterizing and understanding the failure of buparvaquone therapy caused by drug resistance.

DECLARATIONS

Availability of data and materials

The authors declared that all data and materials supporting the results of this study are available in present article.

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Ethical consideration

The authors considered all the ethical concerns, including plagiarism, the double submission, and the originality of the presentation.

Authors' contributions

All authors contribute equally to the research plan. Shehala R. Feidhel, and Howaida H. Abed, collected samples and prepared them for laboratory work. Amer R. Alhaboubi contributed to molecular application and DNA analysis. All authors contribute in writing the manuscript and have agreed to publish the last version and revisions. Amer R. Alhaboubi was the correspondent of the submitted article.

Competing interests

All authors declared no conflict of interest

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Protective Role of *Rosa damascena* Miller Hydroalcoholic Extract on Oxidative Stress Parameters and Testis Tissue in Rats Treated with Sodium Arsenite

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ABSTRACT

Regarding the strong antioxidant properties of Rosa damascene extract, this study aimed to investigate the protective role of Rosa damascene Miller hydroalcoholic petal extract on oxidative stress parameters and testis tissue in rats treated with sodium arsenite. To this end, 30 male rats were divided into five groups, including control, positive control (treated with arsenite), and three groups of patients affected by sodium arsenite with 150 mg/kg, 300 mg/kg, and 450 mg/kg Rosa damascene extract for 34 days by gavage. The animals were then anesthetized, and the blood samples were collected from the heart. The left testis was removed for histopathological studies. The findings revealed that Sodium arsenite in the positive group caused a significant reduction in TAC, testosterone, and serum Luteinizing hormone (LH) and a significant increase in serum Malondialdehyde. In addition, there was no statistically significant difference among the groups regarding the amount of Follicle-stimulating hormone (FSH). Moreover, the consumption of Rosa damascene extract with sodium arsenite caused a significant increase in testosterone, LH, and FSH compared to the positive control group. Histopathological results showed that in the experimental group receiving a dosage of 300 mg/kg b.w and the control group, the number of sperm tubes increased, and the germinal epithelium's thickness was appropriate. Daily treatment with Rosa damascene extract with a dosage of 300 mg/kg b.w for 34 days could improve the changes caused by sodium arsenite and reduce Malondialdehyde levels. Thus, it seems that Rosa damascene hydroalcoholic extract can effectively improve the male reproductive system's function.

Keywords: Oxidative stress, Rats, Rose petals, Sodium arsenite, Testis

INTRODUCTION

In their living and working environments, humans are exposed to agents that can be detrimental to the functioning of the reproductive system. Due to the rapid division of spermatogonia cells, the male reproductive system is very sensitive to many chemicals and physical agents produced by agricultural and industrial activities (Paul and Frazier, 2000).

Among chemical pollutants, arsenic is an element that can be present in mineral waters by dissolving from different soil layers. The concentration of arsenic in groundwater sources of drinking water is among the major global health problems. Inorganic arsenic is found in drinking water in the forms of arsenate (pentavalent) and arsenite (trivalent). The toxicity of arsenite compounds has been reported to be higher than that of arsenate (Mir et al., 2021). In addition, arsenic is used to manufacture herbicides, rodenticides, food preservatives, and even medicines. It is also a carcinogen that can be absorbed in various ways, including the skin, respiratory, and digestive systems, and threaten human and animal health (Wang et al., 2007).

One of the compounds of arsenic is sodium arsenite, which is an odorless and colorless substance. Sodium arsenite is an environmental pollutant that can induce male reproductive system abnormalities through oxidative stress. Arsenic poisoning in laboratory animals weakens Leydig cells' functioning, thereby negatively affecting spermatogenesis (Momeni and Eskandari, 2012). Sodium arsenite induces oxidative stress in various body tissues, including the testes (Akbari, 2022). Oxidative stress and reactive oxygen species (ROS) are considered among the major causes of male infertility. This compound is an oxidizing agent from the group of free radicals that can damage sperm if overproduced (Shi et al., 2004).

Oxidative stress is a pathological process that results from an imbalance between the body's antioxidant defense systems. In this case, the formation rate of free radicals in the body increases and paves the way for the peroxidation and oxidation of lipids, proteins, and nucleic acids (Yamakado et al., 2014).

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Lipid peroxidation (LP) is known as an indicator of OS. The increase in LP is associated with increased malondialdehyde concentration (Yousef et al., 2006). The oxidative stress induction causes fat oxidation and Malondialdehyde production as the main indicator of oxidative stress of lipids and sperm dysfunction (Kubiliene et al., 2021; Salekeen et al., 2022). Due to the presence of fat tissue on the genitals and testes, Malondialdehyde is considered an indicator of oxidative stress (Ahmadi et al., 2011). Natural antioxidants have received more attention because of being safe and have favorable effects in dealing with oxidative stress (Kim et al., 2010). Antioxidants reduce oxidative stress and the amount of testicular damage (Salimnejad et al., 2014). Gole Mohammadi, with the scientific name of Rosa damascena, is a kind of Rose flower in Iran. Essential oil and rose water, as two important product of this plant, has been popular for many years regarding their mystical and economic properties (Shalit et al., 2003).

This plant's extract contains terpene, glycoside, flavonoid, anthocyanin, carboxylic acid, myrcene, vitamin C, kaempferol, quercetin, and geraniol. This plant is a rich source of phenolic compounds such as eugenol and granulol. These compounds have antioxidants. They also inhibit free radicals and have anti-cancer, anti-inflammatory, anti-genetic mutation, and anti-depressant properties. These phenolic compounds also have anti-epileptic properties (Aycı et al., 2005).

Rosa damascene has been long used for food and is considered its essential oil. For this reason, its medicinal uses have been disregarded. On the other hand, research has proven the adverse effects of sodium arsenite on the male reproductive system (Sarkar et al., 2003; Momeni and Eskandari, 2012). Considering the toxicity of sodium arsenite and its negative impact on the male reproductive system and the strong antioxidant properties of *Rosa damascene* extract, this study aimed to investigate the protective role of *Rosa damascene* on oxidative stress parameters and testis tissue of sodium arsenite-treated rats.

MATERIALS AND METHODS

Ethical approval

This research was conducted adhering to ethical principles and with the thesis code 162343080 at the Pet Breeding Center of Shahrekord University.

Study design

A total of 30 adult male Wistar rats aged 8 weeks with an average weight of 225 grams were purchased from the Pasteur Institute of Iran. To adapt to the environment, they were kept in standard conditions (that is the temperature of 22 ± 2℃, a 12:12 light-dark (LD) cycle, and separate standard cages) for 2 weeks with ad libitum access to tap water and special food for laboratory animals (Royan Institute, Iran).

Extraction

Rosa damascene from the gardens of Shahrekord, Iran. Next, after identifying the desired species by a medicinal plant expert from the Research Center for Medicinal Plants. About 500 g of *Rosa damascene* was powdered using a mill and successively extracted three times with 6 L ethanol: water (70:30) at room temperature using a percolator. The extraction process took 72 hours. The obtained liquid extract was filtered through filter paper. Under a vacuum, the extract was evaporated at 35°C until dry using a rotary evaporator (Buchi, Switzerland). The 100-g gummy dark extract was kept in a refrigerator (4°C; Khoshdouni Farahani**,** 2021).

MATERIALS AND METHODS

The rats were carefully weighed and marked into groups before the experiment started. For this purpose, the *Rosa damascene* petal extract has 150, 300, and 450 mg/kg b.w doses. Also, sodium arsenite with a dose of 5 mg/kg b.w were fed to the animals daily and through gavage for 34 days.

In this research, the animals were randomly divided into 5 groups (each with 6 rats):

The first group included healthy controls. The second group included the positive controls exposed to sodium arsenite (5 mg/kg body weight). The third group was treatment group 1 (sodium arsenite + 150 mg/kg body weight of *Rosa damascene* Miller hydroalcoholic petal extract). The fourth group included treatment group 2 (sodium arsenite + 300 mg/kg body weight of *Rosa Damascene* Miller Hydroalcholic Petal Extract). Finally, the fifth group was treatment group 3 (sodium arsenite + 450 mg/kg body weight of *Rosa Damascene* Miller Hydroalcholic Petal Extract).

After the end of the treatment period, the animals were euthanized with ketamine (Pfizer, Germany) at a dosage of 100 mg/kg, and a 2 mL blood sample was taken from their heart (right ventricle) immediately using a 25 gauge needle (Ganguly et al., 2018). The obtained blood was poured into sterile test tubes and centrifuged at a speed of 4000 rpm for 10 minutes. Blood serums were immediately separated, poured into Eppe022ndorf microtubes, and returned to the laboratory to measure biochemical factors (that is Malondialdehyde and total antioxidant capacity). Testosterone, LH,

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and FSH hormones of blood serum were measured using the ELISA method and special kits (Padgin Teb Co, Iran). After taking blood from the animals' hearts, their right testicle was removed from the body and weighed.

Histopathological examination of testis tissue

After washing the testes with physiological serum, they were fixed in 10% formalin for sectioning and histopathological studies. Next, Masson's trichrome staining was performed for samples. Then, the number of seminiferous tubules and spermatogenesis status were checked using an optical microscope (B-510BF, Optika, Italy) by a magnification of ×10 (Kuntsal et al., 2023)

Statistical analysis

The results were analyzed through analysis of variance (ANOVA) and Tukey's tests in SPSS 25 at a significance level of 0.05 was chosen.

RESULTS

The weight of the rats in treatment groups 2 and 3 was almost close to each other and had a significant increase compared with other groups. Also, the weight of the rats in the positive control group and treatment group 1 were almost close to each other and had a significant decrease compared to the other groups ($p < 0.05$). These changes in the healthy control group had a significant decrease compared with the treatment groups 2 and 3 ($p < 0.05$).

The changes in the weight of the testis + epididymis in the treatment groups (2 and 3) had a significant increase compared with the treatment groups 1 and positive control ($p < 0.05$). Also, the testis + epididymis weight in the positive control group had a significant decrease compared with the other groups ($p < 0.05$).

The amount of Malondialdehyde in the positive control group (i.e., the group receiving only sodium arsenite) was higher than that in the healthy control group, indicating a significant difference ($p < 0.05$). There was also a significant difference between treatment groups 1, 2, and 3 ($p < 0.05$) such that treatment group 1 (sodium arsenite + 150 mg/kg body weight of the extract) had an increase in the amount of Malondialdehyde compared with the other two groups ($p < 0.05$).

Moreover, treatment group 2 (dose of 300 mg/kg body weight of the extract) had a decreasing trend in Malondialdehyde compared to the healthy control group, although the difference was nonsignificant. The TAC level significantly increased in the healthy control group compared with the other groups ($p < 0.05$). Moreover, the TAC level of the serum had a significant increase in treatment group 2, compared to treatment groups 1 and 3. However, it decreased significantly in the positive control group compared with the healthy control and treatment groups ($p < 0.05$).

The serum testosterone level significantly increased in the healthy control group, compared with the treatment groups ($p < 0.05$), and in treatment group 2 compared with treatment groups 1 and 3. In addition, the testosterone level significantly decreased in the positive control group compared with the other groups ($p < 0.05$).

As can in seen in Table 1, the serum LH level significantly increased in the healthy control group, compared to the treatment groups ($p < 0.05$). It also significantly increased in treatment group 2 compared with treatment groups 1 and 3 ($p < 0.05$). Notably, the serum LH level had a significant decrease in the positive control group compared with other groups ($P < 0.05$). The serum FSH level had no significant increase in treatment group 2 compared with treatment groups 1 and 3 ($p < 0.05$). However, the serum FSH level had no significant decrease in the positive control group compared with the other groups ($p > 0.05$).

Table 1. Comparison of the mean values of weight and hormonal status in rats treated with sodium arsenite

Traits Mean±SD / Group	MDA (nmol/L)	TAC (nmol/L)	Testosterone (nmol/L)	LH (nmol/L)	FSH (nmol/L)	Rat's weight (g)	Testis and epididymis Weight (g)
Healthy Control	0.13±0.03^{a}	623.00 ±102.50^{b}	1.62 ± 0.29^{c}	0.35 ± 0.06^{c}	0.43 ± 0.21^{a}	237.67 ±4.04^{bc}	1.77 ± 0.13^{bc}
Positive Control	0.24±0.04^{b}	338.30 ± 78.92^{a}	0.52 ± 0.39^{a}	0.11 ±0.03^{a}	0.38 ± 0.13^{a}	208.00 ±19.24^{a}	1.24 ± 0.17^{a}
Treatment 1	0.19± 0.06^{ab}	532.60 ± 41.50^{b}	0.82 ± 0.06^{ab}	0.17 ±0.04^{ab}	0.40 ± 0.07^{a}	210.80±19.25^{ab}	1.35 ± 0.17^{a}
Treatment 2	0.12±0.1^{a}	572.20 ± 41.90^{b}	1.14 ± 0.23^{bc}	0.23 ± 0.06^{b}	0.46 ± 0.15^{a}	248.80 ± 7.79^{c}	1.89 ± 0.19^{b}
Treatment 3	0.13± 0.02^{a}	539.60 ± 49.14^{b}	0.87 ±0.08^{ab}	0.16 ± 0.03^{ab}	0.44 ± 0.11^{a}	247.60 ± 8.62c	1.72 ± 0.19^{b}
p-value	0.000*	0.000*	0.000*	0.000*	0.881	0.000*	0.000*

^{abc} Different superscript letters in a column denote a significant difference between the control group and treatment groups at a significance level of $p \leq 0.05$. TAC: Total antioxidant capacity, FSH: Follicle-stimulating hormone, MDA: Malondialdehyde, LH: Luteinizing hormone, SD: standard deviation

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Pathology results

In the control group, seminiferous tubules have a high density, and germinal epithelium has an appropriate thickness (Figure 1). In the testes (sodium arsenite), seminiferous tubules have separation and ruptures, and the thickness of germinal epithelium has been reduced (Figure 2). In the testes in treatment group 1 (receiving 150 mg of the extract + sodium arsenite), seminiferous tubules have lost their normal shape, and the separation between the cells and the tubes can be seen. The thickness of the gem layer has also decreased compared with the control group (Figure 3). In treatment, group 2 (receiving 300 mg of the extract + sodium arsenite), seminiferous tubules have a relatively high density (Sertoli cells, spermatids, and mature sperm), and germinal epithelium has an appropriate thickness **(**Figure 4**).** In the testis in treatment group 3 (receiving 450 mg of the extract + sodium arsenite), seminiferous tubules have lost their normal structure to some extent, and separation between the tubes can be seen. The thickness of germinal epithelium has also been reduced compared with the control group (Figure 5).

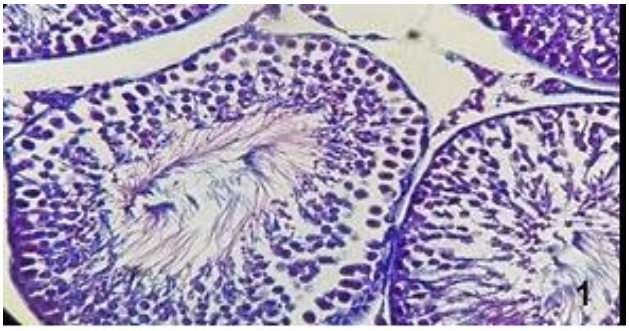

Figure 1. The testis of a rat in healthy control group. Seminiferous tubules have a high density (spermatogonia, mature sperm, and spermatids), and germinal epithelium has an appropriate thickness (Masson's trichrome staining, 100x magnification).

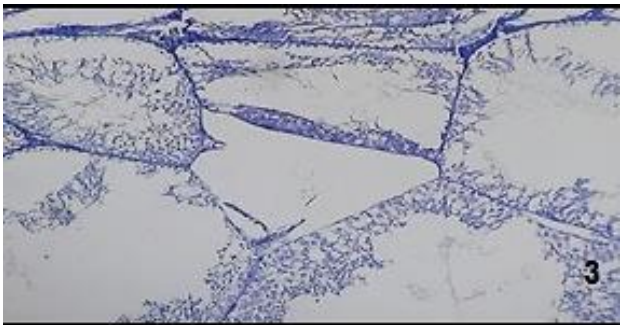

Figure 3. The testis of a rat in treatment group 1 (receiving 150 mg of the extract + sodium arsenite). Seminiferous tubules have lost their normal shape, and the separation between the cells and between the tubes can be seen. The thickness of the gem layer has also decreased compared with the control group (Masson's trichrome staining, 100x magnification

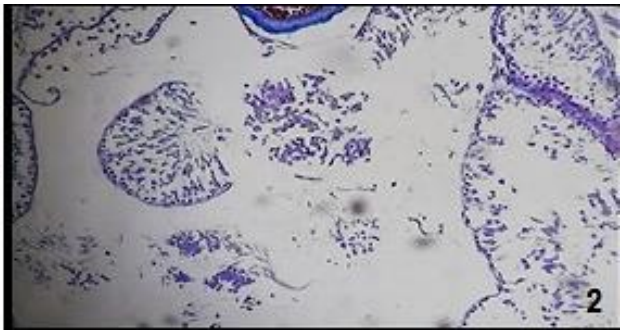

Figure **2.** The testis of a rat in positive control group (sodium arsenite). Seminiferous tubules have separation and ruptures, and the thickness of germinal epithelium has been reduced (Masson's trichrome staining, 100x magnification).

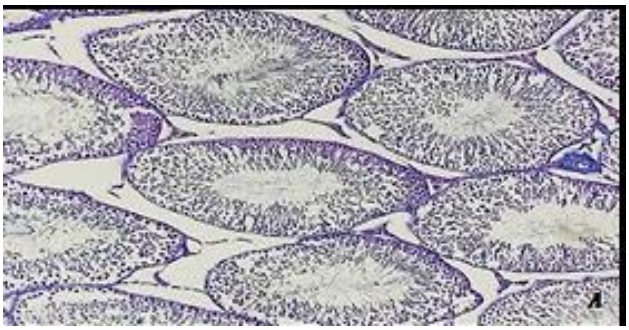

Figure 4. The testis of a rat in treatment group 2 (receiving 300 mg of the extract + sodium arsenite). Seminiferous tubules have a relatively high density (Sertoli cells, spermatids, and mature sperm), and germinal epithelium has an appropriate thickness (Masson's trichrome staining, 100x magnification).

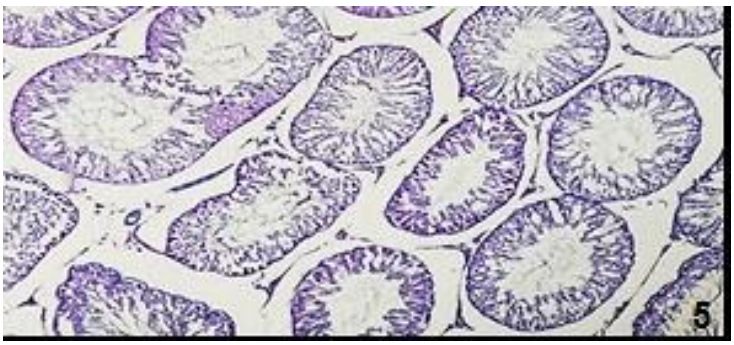

Figure 5. The testis of a rat in treatment group 3 (receiving 450 mg of the extract + sodium arsenite). Seminiferous tubules have lost their normal structure to some extent, and separation between the tubes can be seen. Thickness of germinal epithelium has also been reduced compared with the control group (Masson's trichrome staining, 100x magnification).

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DISCUSSION

There is accumulating evidence regarding the enhanced free ra+dicals generation and oxidation stress induction in animals exposed to inorganic arsenic and ROS-induced toxicity. Research has shown that sodium arsenite has toxic effects on the reproductive system, and its accumulation in the testes and prostate gland causes sperm dysfunction and imbalance in sex hormones (Sarkar et al., 2003; Jana et al., 2006). This effect of sodium arsenite is due to the creation of OS (Agarwal et al., 2004). So far, various studies have been conducted on the effect of plant extracts against LP, reduction of antioxidant defense, and imbalance of gonadotropins and testosterone in male rats. These studies are briefly discussed in the present study.

Hfaiedh et al. (2011) investigated the protective effects of garlic (Allium sativum) extract upon oxidative stress induced by lindane (a white crystalline powder used as an insecticide in agriculture) and related damages in the testes and brain of male rats. According to these authors, the daily injection of fresh garlic at the dose of 1 ml/kg body weight of rats for 30 days returns the lindane-induced changes to a normal state. It reduces the malondialdehyde level (Hfaiedh et al., 2011). These results are consistent with those of the present study and confirm the useful role of these plants as antioxidants. Raji et al. (2019) investigated the effect of hydroalcoholic garlic extract and N-acetylcysteine on fenvalerate-induced OS. The results revealed that fenvalerate in low doses induces OS, and injection of N-acetylcysteine and the hydroalcoholic extract of garlic alone and in combination can improve injuries caused by fenvalerate. The findings of this study are consistent with the present study.

Faraji et al. (2013) examined the effect of the extract of salep tubes on the formation of testis and sexual hormones in mice. They concluded that LH and testosterone expanded in the experimental group compared with the control group ($p < 0.05$). According to this study, the aqueous extract of the salep tubers increases the testosterone and LH hormones and the process of spermatogenesis and strengthens sexual powers. However, further research was recommended to find the mechanism and effect of this substance before it is used as an effective agent in increasing sexual activity and fertility. Jana et al. (2006) studied the effects of chronic exposure to sodium arsenite on testicular activities in adult rats. These authors concluded that treatment with sodium arsenite declined the weight of both testes, the number of epididymal sperm, LH and FSH hormones, and testosterone, and increased plasma corticosterone concentration (Jana et al., 2006).

Anwar and Qureshi (2019) investigated the effect of sodium arsenite on mice testis and epididymal organ cultures in initiating oxidative, biochemical, and genotoxic stress in testis and epididymal tissue cultures in mice. The results showed that testosterone concentrations are significantly reduced after 24 hours of incubation with 50 and 100 micro mols of sodium arsenite concentrations. The exposure of tissue pieces of the testis and epididymis to higher concentrations of arsenic in laboratory conditions induced rapid and immediate metabolic and genotoxic damage.

Shariati et al. (2009) examined the reaction of trifluralin on LH, FSH, and testosterone levels and testis histological changes in adult rats. They concluded that trifluralin passes through the cytoplasmic membrane of sensitive cells in the testis tissue, produces free radicals, and applies OS to them. Hence, they decline the testosterone serum concentration and disrupt the steroidogenesis and spermatogenesis processes. Askaripour et al. (2018) studied the effect of an aqueous extract of *Rosa damascena* on formaldehyde-get toxicity in mice testes. The results revealed that formaldehyde administration significantly reduced the serum testosterone level, testis volume and weight, tubule diameter, and sperm characteristics compared with the control group. Administration of *Rosa damascena* (40 mg/kg b.w) in rats treated with formaldehyde significantly improved serum testosterone level, weight, and histopathological structure of testis, tubule diameter, number of Leydig cells, and epididymal sperm characteristics compared with low doses and the control group (Askaripour et al., 2018).

According to the results of previous studies and the present study, sodium arsenite decreases body weight and testes, gonadotropin and testosterone hormones, and the number of cells in the testis tissue. The level of Malondialdehyde, one of the end products of LP, and a diagnostic tool for infertility analysis, increased after administering sodium arsenite (Zubair et al., 2016). It is worth mentioning that TAC, that is the total antioxidant activity of the body, had a significant decrease in this study in the positive control group compared with the other groups.

Testosterone serum concentration in treatment groups 1, 2, and 3 decreased significantly compared with the control group. Testosterone has direct anabolic effects on protein production in all organs and tissues of the body and increases muscle and bone mass in males (Ganong, 2001). In the groups treated with sodium arsenite, a decrease in testosterone may decline muscle volume and body weight by lowering protein production.

According to previous studies, the physiological concentrations of testosterone (FSH and LH) play an essential role in spermatogenesis (Ganong, 2001). In this study, the administration of sodium arsenite resulted in the reduction of these hormones, which is one of the main factors in the reduction of the number of germ, sertoli, and interstitial cells and degeneration of the testis tissue. Hence, the reduction of testicular weight is not unlikely.

A dose-dependent reduction in plasma and intratesticular testosterone concentration may lead to an increase in arsenic-treated rats due to the inhibition of testicular androgenic enzymes. The reason is that these enzymes are managed to regulate testosterone biosynthesis (Jana et al., 2006). Research has shown that arsenic-induced ROS affects testosterone production by reducing the expression of StAR and Cyp11a1 genes. These genes transfer cholesterol into

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the mitochondria, break the side chain of cholesterol, and convert it into pregnenolone, respectively (Wang et al., 2015; Hu et al., 2020). Additionally, the diffidence of testicular androgenic enzymes in arsenic-treated rats may result from low plasma levels of LH, which is the primary regulator of testicular androgenic enzyme action. The secretion of large amounts of corticoids from the adrenal gland may cause low levels of gonadotropins. Elevated levels of corticosterone have been observed in arsenic-treated animals. Besides, arsenic activates the adrenocortical-hypophyseal stress signaling pathway and increases ACTH secretion from the pituitary gland. An increase in the plasma levels of corticosterone may suppress the sensitivity of gonadotrope cells to GnRH hormones, thereby inhibiting the secretion of FSH and LH hormones. Besides, high levels of ACTH and corticosterone directly inhibit the production and secretion of testosterone by lowering LH receptors on the surface of the testis. Therefore, the reduced process of spermatogenesis decreases the number of sperm (Akbari et al., 2022). In other studies, the sodium arsenite-dependent decrease in plasma gonadotropins has been attributed to low levels of dopamine and high amounts of noradrenaline in the hypothalamus and pituitary. The explanation is that catecholamines are important regulators of the secretion and synthesis of gonadotropins (Jana et al., 2006).

An increase in the malondialdehyde level and a decrease in the total antioxidant level indicate the generation of free radicals and OS. Therefore, the testicular changes in the present study may be associated with oxidative damage caused by sodium arsenite. The hydroalcoholic extract of *Rosa damascene* contains abundant antioxidants, including flavonoid and polyphenolic compounds, with the ability to absorb free radicals (Aycı et al., 2005). The antioxidant effects of the compounds found in this plant have been proven in many studies (Shahriari et al., 2007). Moreover, the other compounds of *Rosa damascene* hydroalcoholic extract include vitamin C, carboxylic acid, tannin, and especially flavonoid and polyphenolic compounds with high antioxidant power, including Kaempferol and Quercetin (Leenen et al., 2000; Loghmani-Khozani et al., 2007).

Based on the histopathological examination of the testis tissue in the different groups of the present study, in treatment group 2 (receiving 300 mg/kg b.w of the extract + sodium arsenite), seminiferous tubules were seen with high density, short distance, and very regular in the testis tissue. All the seminiferous tubules had a round abdomen, a high cell density, and an orderly arrangement. Therefore, a dose of 300 mg/kg b.w of the extract can partially reduce the destructive impacts of sodium arsenite on testis tissue. According to the present study, administrating a dose of 150 mg/kg b.w of the extract + sodium arsenite cannot neutralize the harmful impacts of sodium arsenite. Considering the destructive effects of using a dose of 450 mg/kg b.w of *Rosa damascene* extract + sodium arsenite on testis tissue, using such a dose seems dangerous. Therefore, by applying OS to the testis tissue, sodium arsenite reduces its function and spermatogenesis (Figures 1-5).

The present study indicated that the dose of 300 mg/kg b.w of *Rosa damascene* miller hydroalcoholic petal extract, compared with the samples treated with the dose of 450 mg/kg b.w, is more effective on the OS induced by sodium arsenite. Although the reason for this result is unknown, it seems that antioxidant substances act as prooxidants in certain conditions and cause OS, leading to tissue damage. This factor l the toxicity of the drug in this dose is a question that requires further research.

In the current study, the significant advance in serum testosterone degree and histopathological modification in testes by *Rosa damascene* miller hydroalcoholic petal extract in sodium arsenite-treated rats may be attributed to its antioxidant effect. However, further research is required to clarify the mechanism of action of these compounds on the human reproductive system.

CONCLUSION

The present study showed that the daily administration of *Rosa damascene* Miller hydroalcoholic petal extract at the rate of 300 mg/kg b.w for 34 days returns the sodium arsenite-induced changes to a normal state and reduces the malondialdehyde level. Accordingly, it seems that *Rosa damascene* hydroalcoholic extract can effectively improve the functioning of the reproductive system because of its high antioxidant properties.

DECLARATIONS

Authors' contributions

Shiva Mahdian experimental animals and dosed them with the materials throughout the experiment. Elham Moghtadaei designed the study and critically revised the manuscript. Moghtadaei and Mahdian contributed to the research of the notification data, analyses, and the writing of the final manuscript. The authors confirmed the statistical results and the final draft of the manuscript.

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Conflict of interests

The authors declare no conflict of interest.

Availability of data and materials

The authors can provide all necessary data to the editor upon request without delay.

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Ethical consideration

The authors checked the final draft of the manuscript to remove possible plagiarism and misconduct and prevent double publication/submission and redundancy.

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Protection of Khaki Campbell Ducks against Duck Plague Using an Inactivated Duck Plague Vaccine

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ABSTRACT

Duck plague (DP) or duck viral enteritis is a fatal viral disease of ducks that causes huge economic losses in the duck industry. The present study was performed to determine the immune response and protective efficacy of an inactivated DP vaccine prepared from a local virulent DP virus. A virulent DP virus was obtained from the laboratory repository of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh (Bangladesh). The DP virus (EID_{50} $10^{5.3}$/ml) was inactivated using 0.04% formalin. The alum (40 g/L) was added to the inactivated DP virus as an adjuvant. A total of 60 Khaki Campbell male ducks aged 17 weeks were randomly divided into three groups. Ducks of groups A (n = 20) and B (n = 20) were vaccinated intramuscularly in the breast muscle with 1 ml of inactivated DP vaccine and a live attenuated DP vaccine, respectively. Ducks of group C (n = 20) were kept as unvaccinated control. Booster vaccination was administered at 2 weeks after primary vaccination. Antibody titers of vaccinated ducks were measured at 7, 14, 21, and 28 days post-vaccination (DPV) using a passive haemagglutination (PHA) test. Ducks of both vaccinated and unvaccinated groups were challenged with 1 ml virulent DP virus (EID_{50} $10^{4.3}$/ml) at 28 DPV. Clinical signs, morbidity and mortality, and gross pathological lesions of vaccinated and control ducks were observed for 10 days post-challenge to evaluate the protective efficacy of inactivated DP vaccine. The mean PHA antibody titers of vaccinated ducks of group A at 7, 14, 21, and 28 DPV were 5 ± 0.43, 26 ± 1.71, 43 ± 3.4, and 54 ± 3.28, respectively. Ducks in group B had mean serum PHA antibody titers of 21 ± 1.71, 41 ± 3.28, 52 ± 3.41, and 84 ± 7.25 at 7, 14, 21, and 28 DPV, respectively. No mortality or gross pathological lesions were observed in vaccinated ducks after they were subjected to a challenge infection. Additionally, no significant difference was observed between groups A and B in terms of the challenge infection. The mortality rate of the control group of ducks was 70%. Hemorrhage in the trachea and intestine and necrotic foci in the liver were seen in unvaccinated control ducks (group C). Experimentally developed inactivated DP vaccine induced a protective serum antibody titer and conferred 100% protection against virulent challenge infection up to 10 days observation period.

Keywords: Duck plague, Khaki Campbell, Protective efficacy

INTRODUCTION

The duck plague (DP), also known as duck virus enteritis, is a viral disease of ducks worldwide, including Bangladesh, India, China, and Egypt (El-Tholoth et al., 2019; Neher et al., 2019; Khan et al., 2021; Liang et al., 2022). The causal agent of DP is a double-stranded DNA virus that belongs to the family Herpesviridae (Dhama et al., 2017). This viral infection affects both domestic ducks and wild waterfowl and is extremely contagious and fatal in nature (Kaleta et al., 2007). Its impact is significant, leading to economic losses both in broiler and layer duck farms (Islam et al., 2021).

In Bangladesh, the DP virus was first isolated and identified by Sarker (1980). Outbreaks of DP occur almost every year between March and June in Bangladesh (Sarker, 1980; Hoque et al., 2010). Khan et al. (2018) reported 55.86% mortality due to DP outbreaks in Bangladesh. Several investigators isolated and characterized the DP virus from natural disease outbreaks in Bangladesh (Islam and Khan, 1995; Akter et al., 2004; Ahamed et al., 2015).

Two types of vaccines that can be used to immunize ducks against DP include live attenuated and inactive DP vaccines (Shawky and Sandhu, 1997; Kulkarni et al., 1998). The immune system of duck can recognize both live and inactivated viral antigens and mount immune response. Live attenuated DP vaccine is prepared by attenuating a wild type of DP virus. It mainly induces a cell-mediated immune response and confers adequate protection against DP virus infection (Lian et al., 2010; Huang et al., 2014). This vaccine is routinely used in vaccination programs against DP. In order to be effective live attenuated DP vaccine requires a cold chain during its storage and transport (Khan et al., 2018).

An inactivated DP vaccine contains killed viruses which may still have pathogen-recognition patterns and can induce an antibody-mediated immune response. This vaccine provides shorter-term protection and requires booster doses for long-term immunity (Plotkin, 2008). The killed vaccine does not require a cold chain and has the advantage of using

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developing countries (Melnick, 1978). It can be administered during disease outbreaks. The adjuvant is used in the inactivated vaccine to increase immunogenicity, facilitating higher and longer-lasting immunity. Inactivated DP vaccine conferred 100% protection against the virulent challenge of the DP virus in vaccinated ducks (Shawky and Sandhu, 1997). Soma et al. (2018) prepared an inactivated DP vaccine and tested its antibody response in Khaki Campbell ducklings. The protective efficacy of this inactivated DP vaccine has not been studied in ducks following a challenge infection with the virulent DP virus.

Most European countries and the USA use both live attenuated and killed DP vaccines to prevent DP in broiler ducks and swans (Shawky and Sandhu, 1997; Shawky et al., 2000). Live attenuated DP vaccines produced by the Livestock Research Institute (LRI) or imported from foreign countries are used to vaccinate ducks against DP in Bangladesh. Many commercial DP vaccines yielded an inadequate immune response (Kulkarni et al., 1998). Vaccination failure may result if the seed virus used for vaccine preparation is not antigenically matched with the circulating virus. Although the DP virus is a single antigenic type, vaccination failure is reported (Das et al., 2009; Khan et al., 2018). The inability to maintain a cold chain for live attenuated DP vaccine during its storage and transport might be one reason for vaccination failures in Bangladesh (Khan et al., 2018). There is a need to develop an inactivated DP vaccine since it does not require a cold chain. In some countries where maintaining the cold chain for live DP vaccine during transportation and storage is not feasible, the inactivated DP vaccine may be a suitable replacement for the attenuated live DP vaccine. This present research aimed to develop an inactivated DP vaccine using a virulent local DP virus isolates and to determine antibody response and the protective efficacy of inactivated DP vaccine in the Khaki Campbell duck.

MATERIALS AND METHODS

Ethical approval

The experiments related to the efficacy trial of the DP vaccine and challenge infection with virulent DP virus in Khaki Campbell duck were conducted according to the guidelines of the Animal Welfare and Experimental Ethics Committee of Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh(protocol approval number: AWEEC/BAU/2020/08) and WOAH (2008).

Ducks

Day-old Khaki Campbell (*Anas platyrhynchos domesticus*) male ducklings (n=60) were obtained from a commercial duck farm at Mymensingh, Bangladesh. Parent flocks were vaccinated against DP and duck cholera vaccines. The DP vaccine was made from an attenuated strain of DP virus manufactured by the LRI, Dhaka, Bangladesh. The duck cholera vaccine was produced from the inactivated virulent strain of *Pasteurellamultocida*manufactured by the Livestock and Poultry Vaccine Research and Production Center (LPVRPC), BAU, Mymensingh, Bangladesh. The ducklings were free from diseases confirmed by a veterinarian's physical examination. Ducks were reared for six months from August 2019 to February 2020 in an isolated experimental animal shed at the Department of Microbiology and Hygiene, BAU, Mymensingh, Bangladesh, and supplied with commercial feed (Nourish poultry feed, Dhaka, Bangladesh) three times daily and water *ad libitum*. A veterinarian regularly evaluated the health conditions of ducks.

Virus

A local virulent DP virus was obtained from the laboratory repository of the Department of Microbiology and Hygiene, BAU, Mymensingh (Bangladesh). The virus was revived into 10 days-old embryonated duck eggs through the chorioallantoic membrane (CAM) route for six passages (Ahamed et al., 2015). The stock DP virus was stored in a small aliquot in a 5 ml screw-capped vial at -86°C in the lab repository. The class II A2 biosafety cabinet (Thermo Scientific, Waltham, MA, USA) was used to inoculate DP virus into the embryonated duck eggs. The stock DP virus was previously isolated from a natural outbreak of DP (Islam et al., 2021) and was used to prepare inactivated DP vaccine and challenge infection. The EID_{50} of the DP virus was determined by the standard procedure (Kulkarni et al., 1998).

Vaccines

The DP virus (EID_{50} $10^{5.3}$/ml) was inactivated by 0.04% formalin on a shaker incubator at 37℃ for 24 hours. Virus inactivation was confirmed by three successive blind passages in the 10-day-old embryonated duck eggs. The sterility of inactivated DP vaccine was checked according to the method described by Igomu et al. (2020). Alum adjuvant (0.04 g/ml) was added to the inactivated DP virus suspension and mixed properly on a shaker incubator at 37℃ for 2 hours (Gupta and Rost, 2000; Aguilar and Rodriguez, 2007). A live attenuated DP vaccine(batch no. 04/2019) manufactured by LRI, Dhaka, Bangladesh, was used as a positive control.

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Experiment design

A total of 60 healthy Khaki Campbell ducks aged 17 weeks were randomly divided into three groups (A, B, and C) and reared in three separate houses for 8 weeks from January to February 2020. Serum samples were tested by passive haemagglutination (PHA) test to verify that ducks were free of antibodies against the DP virus. Ducks were adopted in the animal house facilities for one week prior to the experiment. Ducks of group A (n=20) and B (n=20) were vaccinated intramuscularly (IM) at the breast muscle with 1 ml of inactivated DP vaccine and 1 ml of live attenuated DP vaccine, respectively, at 17 weeks of age. A booster dose of the same vaccine was administered at 19 weeks of age (Table 1). Ducks of group C (n=20) were kept as unvaccinated control. Blood samples were collected from the wing vein of all vaccinated and control ducks at 0, 7, 14, 21, and 28 days post-vaccination (DPV) using a 5 ml disposable plastic syringe (JMI Syringe and Medical device, Cummilla, Bangladesh). Sera were separated from blood samples, and antibody titers in sera were determined using the PHA test (Soma et al., 2018). Ducks of all groups (A, B, and C) were challenged by injecting 1 ml of virulent DP virus (EID_{50} $10^{4.3}$/ml) through IM route at 21 weeks of age (Table 1). The challenged ducks were observed twice daily for clinical signs of DP, such as abrupt death, extreme thirst, partial paralysis, and watery, greenish diarrhea (Dhama et al., 2017). Clinical statuses were indicated on the lines of intravenous /intracerebral pathogenicity index (PI) as described in Poultry Biologics National Research Council (NRC, 1963). The clinical manifestation of DP was recorded to calculate PI (Kulkarni et al., 1998). The scores of clinical manifestations of DP are shown in Table 2. The postmortem examination was done for vaccinated and controlled ducks. The live ducks were killed by disarticulation of the head at the atlantooccipital joint without anesthesia (Charlton et al., 2000). Dead ducks were placed on a surgical tray. A longitudinal incision was made through the skin of the neck to the thoracic inlet. Trachea was removed and examined after giving a longitudinal incision. A transverse incision was made through the posterior part of the abdominal muscles. On each side, the incision was given through the costochondral junction. The ventral abdominal wall and breast were removed as one piece. Visceral organs such as the liver, spleen, and intestine were removed. Gross pathological lesions such as hemorrhage in the trachea, intestine, focal necrosis in the liver, and splenomegaly were recorded during postmortem examination.

Table 1. Experimental design to determine the immune response in Khaki Campbell duck from August 2019 to February 2020 at the Bangladesh Agricultural University, Mymensingh, Bangladesh

Operation	Age of ducks (Weeks)	Dose (ml)	Groups		
			A	B	C
Pre-vaccination serum antibody titre	16		20	20	20
Primary vaccination	17	1 ml	20	20	ND
Booster vaccination	19	1 ml	20	20	ND
Challenge infection	21	1 ml	20	20	20

ND: Not done

Table 2. Clinical manifestation of duck plague with scoring factor in Khaki Campbell ducks aged 21 weeks at the Bangladesh Agricultural University, Mymensingh, Bangladesh

Case	Clinical evidence of duck plague	Scoring factor
1.	Death	3
2.	Acute lesions	2
3.	Chronic lesions	1
4.	Normal	0

Protective efficacy

Vaccinated ducks (group A and group B) and unvaccinated control ducks (group C) were challenged with virulent DP virus, and mortality was evaluated for 10 days post-challenge. The protective efficacy, also known as a preventable fraction (PF) of the vaccine, was calculated using the following method described by Tizard (2004).

PF=(% of control dying-% of vaccinated dying)/% of control dying

Pathogenicity index

The PI was calculated using the following method described in Poultry Biologics (National Research Council, 1963; Kulkarni et al., 1998).

PI = Total score/Total number of observations

Vaccinated ducks (groups A and B) and unvaccinated control ducks (group C) were challenged with 1 ml (IM) of virulent DP virus. Clinical statuses, such as death, severe disease, mild disease, and no disease of challenged ducks were monitored for 10 days post-challenged. The following factors were considered while calculating the PI clinical scores for the ducks in each group. Ducks with a score of 3 were dead, 2 had a serious disease, 1 had a slight disease, and 0 were healthy and active. Data from 10 days were combined to produce a sum multiplied by a scoring factor. The total score obtained was divided by the total number of observations to determine the PI.

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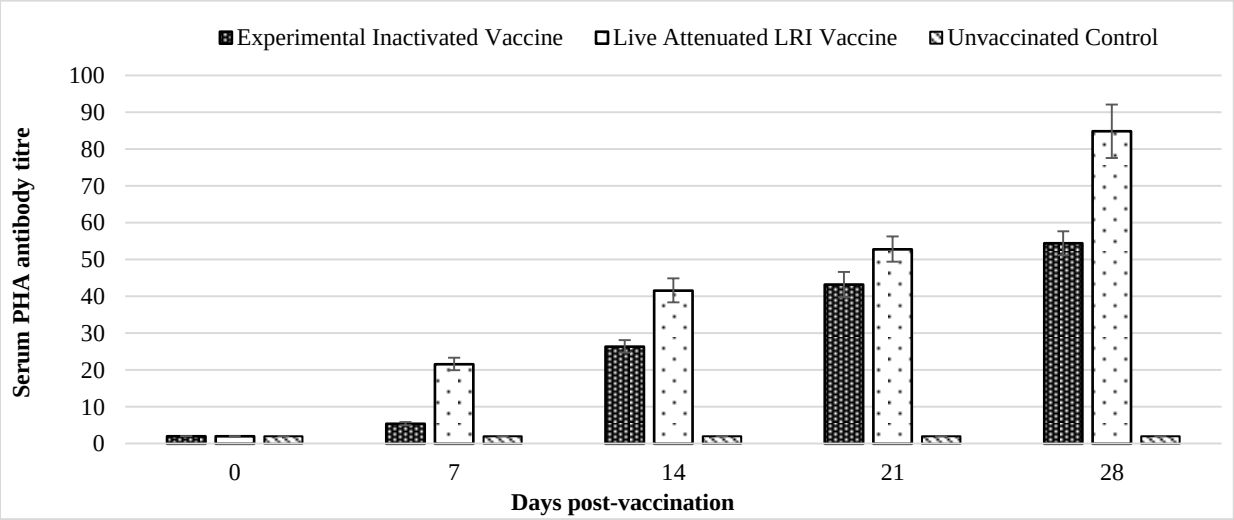
Statistical analysis

Results of the mean PHA serum antibody titer of vaccinated ducks were analyzed using student's t-test and chi-square tests for statistical significance using the statistical package for social science (SPSS) version 25 for Windows 10. A p-value of ≤ 0.05 was considered significant. Serum antibody titers have been presented as mean ± standard error (SE).

RESULTS

Serum antibody titer

Pre-vaccination log2 serum PHA test antibody titer of all ducks in groups A, B, and C was 2. The log2 serum PHA test antibody titer (mean ± SE) of experimentally developed inactivated vaccinated ducks (group A) were 5 ± 0.43, 26 ± 1.71, 43 ± 3.4, and 54 ± 3.28 at 7, 14, 21, and 28 DPV. On the other hand, the log2 mean serum PHA test antibody titer for live attenuated vaccinated ducks (group B) were 21 ± 1.71, 41 ± 3.28, 52 ± 3.41, and 84 ± 7.25 at 7, 14, 21, and 28 DPV. The serum PHA antibody titer(mean ± SE) of the unvaccinated control ducks (group C) remained 2 ± 0 before the challenge experiment. A statistically significant difference in serum antibody titers ($p < 0.05$) was observed in vaccinated ducks (groups A and B) at 7, 14, 21, and 28 DPV when compared to unvaccinated control.



Graph 1. The mean passive haemagglutination (PHA) antibody titer of vaccinated and unvaccinated Khaki Campbell ducks at days 0, 7, 14, 21, and 28 post-vaccination at 17, 18, 19, 20, and 21 weeks of age, respectively, from January 2020 to February 2020 at the Bangladesh Agricultural University, Mymensingh, Bangladesh. Antibody titers are reported as mean ± standard error (SE). A statistically significant difference was found in serum antibody titer between the experimental inactivated vaccine and live attenuated vaccine ($p < 0.05$).

Pathogenicity indices

Vaccinated ducks (groups A and B) showed mild clinical signs of DP, such as inappetence and lethargy after challenge infection with local virulent DP virus with PI of 0.15 (Table 3) and 0.15 (Table 4), respectively. On the contrary, control ducks manifested clinical signs at 48 hours post-challenge with PI 2.70 (Table 5). The clinical signs of watery diarrhea, weight loss, depression, and loss of appetite were observed in the sick ducks.

Preventable fractions

Ducks immunized with inactivated DP vaccine (group A) and live attenuated DP vaccines (group B) were 100% protective against challenge infections (Table 6). In the unvaccinated control ducks (group C), 70% mortality was observed following challenge infection. The PF of both inactivated and live attenuated DP vaccines was 100% (Table 6).

Table 3. Calculation of pathogenicity index of 21-week-old Khaki Campbell ducks vaccinated with experimentally developed inactivated duck plague vaccine in February 2020 at the Bangladesh Agricultural University, Mymensingh, Bangladesh

Clinical evidence of DP	Days of observation										Sum × Scoring factor	Total Scores	PI index (total score/total number of observations)
	1	2	3	4	5	6	7	8	9	10			
Death	0	0	0	0	0	0	0	0	0	0	0 × 3	0	
Acute sign	0	0	0	0	0	0	0	0	0	0	0 × 2	0	
Chronic sign	0	0	0	0	0	0	1	2	0	0	3 × 1	3	0.15 (3/20)
Normal	2	2	2	2	2	2	1	0	2	2	17× 0	0	

DP: Duck plague, PI: Pathogenicity Index

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Table 4. Calculation of pathogenicity index of old Khaki Campbell ducks aged 21 weeks vaccinated with live attenuated duck plague vaccine at Bangladesh Agricultural University, Mymensingh, Bangladesh

Clinical evidence of DP	Days of observation										Sum × Scoring factor	Total Scores	PI index (total score/total number of observation)
	1	2	3	4	5	6	7	8	9	10			
Death	0	0	0	0	0	0	0	0	0	0	0 × 3	0	
Acute sign	0	0	0	0	0	0	0	0	0	0	0 × 2	0	
Chronic sign	0	0	0	0	0	0	0	1	1	1	3× 1	3	0.15 (3/20)
Normal	2	2	2	2	2	2	2	2	1	1	18× 0	0	

DP: Duck plague, PI: Pathogenicity Index

Table 5. Calculation of pathogenicity index of unvaccinated Khaki Campbell aged 21 weeks at Bangladesh Agricultural University, Mymensingh, Bangladesh

Clinical evidence of DP	Days of observation										Sum × Scoring factor	Total Scores	PI index (total score/total number of observations)
	1	2	3	4	5	6	7	8	9	10			
Death	0	0	0	4	3	4	2	1	0	0	14 × 3	42	
Acute sign	0	0	2	3	1	0	0	0	0	0	6 × 2	12	
Chronic sign	0	0	0	0	0	0	0	0	0	0	0 × 1	0	2.70 (54/20)
Normal	2	2	2	0	0	0	0	0	0	0	6× 0	0	

DP: Duck plague, PI: Pathogenicity Index

Table 6. The conferred protection in vaccinated Khaki Campbell ducks following challenge infection with the virulent duck plague virus in Bangladesh Agricultural University, Mymensingh, Bangladesh

Experimental group (n)	Number of dead (%)	Number of survived birds (%)	Preventable fraction of vaccine
A (20)	0 (0)	20 (100)	100%
B (20)	0(0)	20 (100)	100%
C (20)	14 (70)	6 (30)	NA

A: Experimentally develop inactivated duck plague vaccine, B: Live attenuated duck plague vaccine, C: Unvaccinated control; NA: Not applicable

Gross lesions

Gross postmortem lesions observed in unvaccinated control ducks were hemorrhagic annular bands in the trachea, hemorrhagic enteritis in the intestine, white foci in the liver, and splenomegaly (Figure 2). No postmortem lesions were found in vaccinated ducks (Figure 2), and they survived against the challenge of infection and conferred 100% protection.

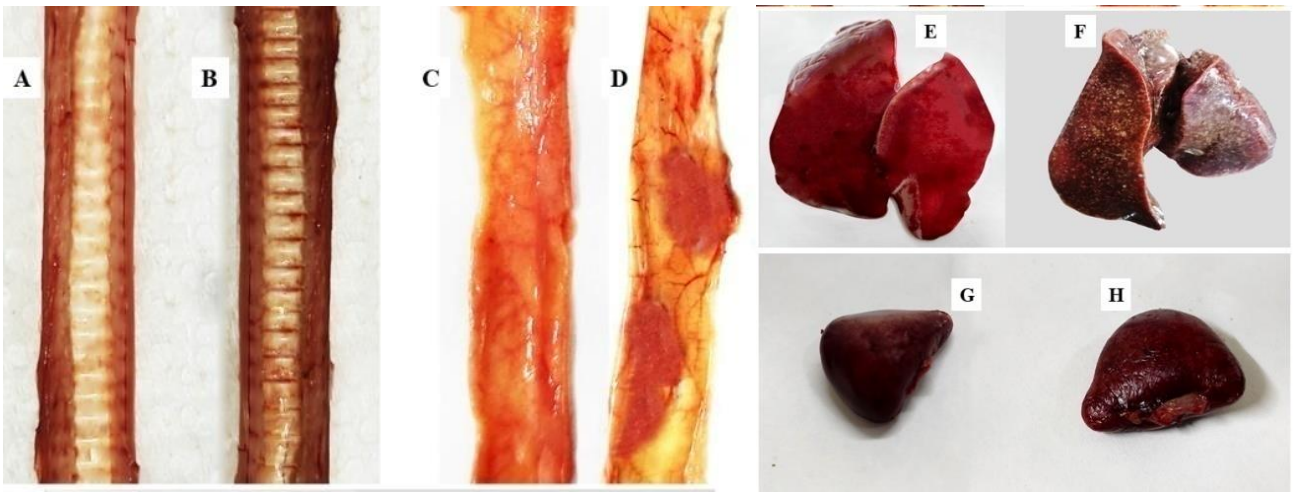

Figure 1. Gross pathological lesions of trachea, intestine, liver, and spleen in vaccinated and unvaccinated control ducks. No lesions were seen in the trachea (A), intestine (C), liver (E), and spleen (G) of experimentally developed inactivated duck plague vaccinated ducks. On the contrary, annular hemorrhagic bands in the trachea (B), hemorrhage in the intestine (D), multiple white necrotic foci in the liver (F), and splenomegaly (H) were observed in unvaccinated control ducks.

DISCUSSION

The duck plague inflicts vast mortality and morbidity in the poultry industry of Bangladesh (Khan et al., 2021). Live attenuated vaccines produced by LRI, Mohakhali, Dhaka, and some private companies are used to vaccinate ducks to control the duck plague in Bangladesh. However, the live attenuated vaccine induces an adequate immune response against the DP virus. Some drawbacks of live vaccines include the reversion of live attenuated viruses into virulent form in the natural host and the lack of heat stability under field conditions (Osman et al., 2021; Ravikumar et al., 2022).

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Thus, it is urgent to develop a DP vaccine that is suitable to use under the field condition of Bangladesh. Several studies indicated that the inactivated duck plague vaccine was protective and advantageous, compared to the live attenuated vaccine (Shawky and Sandhu, 1997). Room temperature is enough to store and can be used in a disease outbreak episode as an emergency vaccination. In Bangladesh, maintaining the cold chain of live vaccines is very difficult, often resulting in vaccination failure (Khan et al., 2018). This problem can be overcome by using an inactivated vaccine. In this study, an attempt was undertaken to evaluate the protective efficacy of an experimentally developed inactivated DP vaccine using local isolate.

It is generally accepted that vaccines produced from local DP virus isolate confer adequate protection against field viral infection (Soma et al., 2018). This study used a well-characterized DP virus isolated from a field outbreak (Islam et al., 2021) to produce the experimentally developed inactivated DP vaccine. The vaccine should have a virus titer of not less than $10^{2.0}EID_{50}$/ dose when tested at any time before the expiry date (ASEAN, 2018). In this study, EID_{50} of the local isolate was fixed to $10^{5.3}$/ml for preparation of inactivated vaccine since the recommended concentration of DPV in the vaccine should be at least EID_{50} 10^3 (Hossain et al., 2005; WOAH, 2008).

Alkylating agents such as formalin and β-propiolactone are widely used in vaccine preparation (Chowdhury et al., 2015). Both can inactivate the virus via the chemical reaction with viral capsid proteins and nucleic acids. However, formalin is a cheaper disinfectant, and a study revealed that a formalin-inactivated vaccine produced a higher serum antibody titer than β-propiolactone inactivated antigen (Chowdhury et al., 2015). Soma et al. (2018) used 0.12% formalin to inactivate the virus for DP vaccine preparation. Viruses inactivated by formalin cannot be reverted into virulent form. Large amounts of antigen are essential to provoke an adequate antibody response. As formalin has a significant disadvantage, uncontrolled use may damage antigens enough to modify immunogenicity to elicit cell-mediated immune responses, resulting in a short-duration immune response (Burrell et al., 2016). In this experiment, 0.04% formalin was used to inactivate the DP virus. Some used 0.04% formalin to inactivate poultry viruses to produce viral antigens (King, 1991; Elveborg et al., 2022).

An adjuvant enhances the immune response to inactivated vaccine (Edelman, 1980). It enhances phagocytosis, antigen depot, and prolongs immune response by slowly releasing antigens (Wilson et al., 2017). In this study, alum was used as an adjuvant. It is also known as potassium alum or aluminum sulfate, chemically formulated as $KAl(SO_4)_2 \cdot 12H_2O$. Antigens are absorbed into aluminum salts resulting in high concentrations of antigen at the injection site, which are taken up by antigen-presenting cells (HogenEsch, 2002). Alum reacts like a mild irritant, causing the employment of leukocytes required to produce an immune response to the injection site. Aluminum compounds can enhance the immune response by activating complement, stimulating dendritic cells, and releasing chemokines. Several investigators used alum to produce the killed vaccine (Hossain et al., 2004; Wang et al., 2021).

In this study, ducks were vaccinated through the IM route at 17 weeks of age. The muscles of the ducks have abundant blood circulation, making it easier for the body to absorb the drug rapidly. In this study, 1 ml of the vaccine was used to immunize ducks through the IM route. Subcutaneous and IM are the most preferred routes for vaccination of inactivated vaccines. These routes offer a slow release of vaccines from the vaccination site (Kayesh et al., 2008). A booster vaccination is recommended for the inactivated vaccine to prolong the duration as well as increase the antibody titer of the vaccine (Shawky and Sandhu, 1997). In this study, booster vaccination was administrated, which induced statistically significant antibody titer.

Antibody titers of vaccinated ducks were measured by the PHA test. This test is commonly used to measure DP vaccine antibody response (Akter et al., 2004). However, the lack of specificity becomes particularly noticeable at low antibody titters due to the assay's inability to distinguish between biologically active and non-neutralizing antibodies (Roper et al., 2013).

The inactivated vaccine induced the highest antibody titer (54 ± 3.36) at day 28 post-vaccination. Hossain et al. (2005) and Kayesh et al. (2008), respectively, reported protective serum PHA antibody titers of 115.2 ± 12.8 and 57.60 ± 6.40 for the duck plague vaccination. Ducklings with PHA titers 22 ± 0.7 exhibited 100% resistance to the virulent DP virus challenge, according to Konwar et al. (2020). In this study, antibodies present in vaccinated ducks might have neutralized the virulent DP virus following challenge infection, which results in the protection of vaccinated ducks, compared to unvaccinated control.

The protective efficacy of the vaccine was calculated by challenge experiment. The experimentally developed vaccine was 100% protective against virulent DPvirus infection. A vaccine is considered adequate if it protects at least 80% of the challenge infection (Islam et al., 2009). The comparison of the PI of the experimentally developed inactivated DP vaccine and live attenuated DP vaccine indicated that both vaccines induced similar protection against virulent challenge infection. No pathological lesions were recorded in the vaccinated ducks, compared to the unvaccinated control following the challenge. Neutralization of the virus by the antibody of vaccinated ducks might prevent the localization of the DPvirus into the lymphoid tissues of vaccinated ducks.

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CONCLUSION

Data from this study suggest that the inactivated DP vaccine was effective against virulent DP virus infection in the current study condition and could be used as a suitable alternative to the live attenuated vaccine under the field condition of Bangladesh. However, the field trial for developing the administration of inactivated DP vaccine should be carried out on duck farms to evaluate its protective efficacy.

DECLARATIONS

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Availability of data and materials

The datasets generated for the current study are available from the corresponding author upon request.

Ethical consideration

All authors carefully checked the ethical issues such as plagiarism, misconduct, data fabrication, falsification, manuscript redundancy, and duplicate publication or submission.

Competing interests

The authors declare no conflict of interests.

Authors' contributions

Tanvir Ahamed designed and conducted the experiment, analyzed data, and wrote the manuscript. Papia Sultana collected samples and conducted an experiment. Md. Zaminur Rahman interprets the results of the postmortem examination and analyzed the data. Palash Bose conducted laboratory work and analyzed data. Mohammad Rafiqul Islam designed the experiment and wrote the manuscript. Mst. Minara Khatun designed the experiment and edited the manuscript. Md. Ariful Islam conceptualized and designed the experiment, critically analyzed the data, and wrote and revised the manuscript. All authors read and approved the last version of the manuscript.

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Effects of Commercial and Homemade Extenders on Post-thaw Sperm Quality and Fertility of Semen from Ethiopian Indigenous Horro Chicken Breed

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ABSTRACT

Cryopreservation of spermatozoa represents an important strategy for *in vitro* programs designed for the conservation of the genetic material of livestock populations. The objective of this study was to evaluate the effects of homemade tris-egg yolk-based and commercial poultry semen extenders on post-thaw sperm quality, fertility, and hatchability of semen from the Ethiopian Indigenous Horro chicken breed. A total of 30 roosters were used for semen collection, and 160 adult hens were inseminated artificially. The collected, qualified, and pooled semen samples were divided into three groups. Each semen sample was diluted with a homemade tris-egg yolk-based extender, Dimethyl-formamide commercial extender, and Commercial Beltsville Poultry Extender. Each extended semen was further divided into 20 sterile tubes as replicates. The extended semen samples were cryopreserved in liquid nitrogen using standard procedures. Changes in post-thaw spermatozoa mass and progressive motility, *in vitro* viability, morphological abnormality, fertility, and hatchability were evaluated. The post-thaw evaluation showed a decrease in the mass and progressive motility, morphologically normal spermatozoa, and an increase in dead spermatozoa and spermatozoa with bent necks, compared to fresh semen. There were significant differences in progressive sperm motility, motility, and *in vitro* viability between commercial and homemade cryoprotectants. However, no significant difference was observed in mass motility across the extenders. The commercial Dimethyl-formamide extender was found to be the most suitable regarding the proportion of morphologically normal sperm and *in vitro* viability rate of cryopreserved sperm samples. There were no significant differences across all treatments in terms of fertility and hatchability rate. However, there was a significant difference between the control treatment and commercial extenders in terms of fertility and hatchability. The findings indicated favorable outcomes for a tris-egg yolk-based extender that was prepared locally with regard to the cryopreservation of poultry semen. Additional investigations are recommended to enhance the fertility and hatchability of the semen.

Keywords: Cryopreservation, Horro, *In vitro* viability, Morphology, Motility, Semen, Sperm

INTRODUCTION

Cryopreservation of semen is an efficient method for the *ex-situ* management of avian genetic resources (Ehling et al., 2012). However, the success of cryopreservation largely depends on the choice of semen extender used. Therefore, evaluating and identifying the most suitable semen extender for the cryopreservation of semen from the Ethiopian Horro chicken breed is essential to ensure the preservation of its genetic resources. Extenders can be defined as buffered salt solutions used to prolong the viability of good-quality semen. The main advantage of commercial extenders is their availability as well as standardized composition and application (Petriˇcáková et al., 2022). Cryopreservation of spermatozoa could play a crucial role in genetic resource conservation as the conservation of poultry genetic resources by the living flock is costly (Han et al., 2005).

Many factors, such as the different types of cryoprotectants (CPAs), packaging, and cooling rates, could affect the quality of cryopreserved semen (Gerzilov, 2010). Glycerol is the most widely used cryoprotectant for cryopreservation of chicken semen. Continuous studies and improvements in the use of glycerol in semen extenders may enhance the fertility of post-thawed semen (Zong et al., 2022). Practices for *ex-situ* preservation of endangered breeds have been studied for the past decades (Thélie et al., 2019). Genetic stocks of chicken genetic diversity in cryobanks have been developed using cryopreservation of semen and primordial germ cells, and gonadal tissues. Due to its non-invasive nature, the cryopreservation of semen stays the preferred method (Thélie et al., 2019). Several protocols have been developed to cryopreserve semen from chickens. However, the results obtained gave a highly variable success rate. Several studies have reported that Glycerol-based CPAs maintain the quality of spermatozoa (Seigneurin and Blesbois, 1995; Partyka et al., 2012; Miranda et al., 2017). Therefore, this study aimed to assess post-thaw microscopic qualities and fertility of cryopreserved semen using locally prepared Glycerol-based CPA and commercial CPAs of semen

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collected from Horro chickens.

MATERIAL AND METHODS

Ethical approval

The present study followed institutional guidelines for humane animal treatment and complied with relevant legislation from Addis Ababa University College of Veterinary Medicine, Bishoftu, Ethiopia.

Animal management

For semen collection, a total of 30 adult Horro cockerels with an average age of 40 weeks and an average body weight of 1.7 kg were used. The roosters were kept separately from the hens and trained for semen collection by abdominal massage technique for 2 weeks. For artificial insemination, 160 adult hens with similar age groups were used. The experimental chickens were purchased from Debrezeit Agricultural Research Center, Bishoftu, Ethiopia. The roosters and the hens were kept in a deep litter system with a depth of 12 cm in pens that had a total area of 30 m^2. They were fed a breeder ration containing 17% crude protein and 2800 Kcal/Kg energy. Feed was provided twice a day at an amount of 110 gm/bird/day and water was provided *ad libitum*. All experimental chickens were vaccinated for major diseases, including Newcastle, Marek's, Gumboro, fowl pox, and fowl typhoid. The chickens were given a 2-week adaptation period in the experimental environment before sample collection and artificial insemination.

The average temperature and humidity of the chicken house were 22°C and 41%, respectively. The lighting conditions used in the experiment were 16 hours for the hens during the laying period and 12 hours for the roosters.

Extender preparation

The homemade extender used in this study was a Glycerolized tris-egg-yolk-based extender. Semen diluents were prepared by mixing tris (base), citric acid, fructose, and chicken egg yolk. Antibiotic 25 mg of gentamicin (Wockhardt Ltd, UK) was added into the extender at room temperature of 25°C. The composition of diluents is presented in Table 3.

Table 1. Breeder ration formula used during the experiment

Feed ingredient	Inclusion rate (%)
Corn	52
Soy cake	10
Meat and bone meal	6
Wheat bran	15
Noug cake	9
Limestone	6
Breeder premix	0.5
Lysine	0.1
Methionine	0.1
Molasses	1
Salt	0.3

Breeder premix: Industrial, well-balanced premix that ensures fertile, hatching eggs and ultimately strong chicks. It contains vitamins and minerals.

Table 2. Vaccination schedule used for Horro chicken

Age	Vaccination against	Application
Day 1	Marek	Subcutaneous (neck)
Day 2	Newcastle disease	Eye drop
Day 7	Gumboro	Drinking water
Day 14	Newcastle (Lasota)	Drinking water
Day 18	Gumboro	Drinking water
Week 6	Newcastle (Lasota)	Drinking water
Week 8	Fowl typhoid	Injection
Week 9	Deworming	Drinking water
Week 10	Fowl pox	Wing stab
Week 14	Fowl typhoid	Injection

The vaccines originated from the National Veterinary Institute, Bishoftu, Ethiopia.

Table 3. Contents of the homemade extender

Contents	Amount
Tris (base)	2.42 gm
Citric acid	1.48 gm
Fructose	4 gm
Chicken egg-yolk	20 % v/v
Gentamicin	200k IU
Double distilled water	100 ml

pH was adjusted to 6.7

Semen collection and initial evaluation

Semen was collected using the Quinn and Burrows abdominal massage technique developed. The semen was collected with a sterile tube. Two ejaculates were collected from the roosters at Debre Zeit Agricultural Research Center, Poultry Farm, Bishoftu, Ethiopia. After collection, the semen was maintained in a water bath at 37°C and subjected to pre-freeze evaluation

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on site. Fresh semen collected was evaluated for semen volume, color, pH, sperm concentration (bill/ml), motility (%), morphological abnormality (%), and live percent. Qualifying ejaculates were pooled to get sufficient semen for a replicate having motility > 60%, live percent > 70%, and morphological abnormality < 30% for further processing (Getachew et al., 2015).

Semen processing for cryopreservation

Qualifying ejaculates (pearly-white, free of any fecal contamination, above 0.3 ml, sperm motility of above 60%, sperm concentration of above 1 × 109 sperm cells/ml) were used for cryopreservation. The pooled semen was divided into three equal volumes and diluted with E1 (homemade extender), E2 (commercial extender Avian Semen Diluent, Minitube International, Tiefenbach, Germany), and E3 (commercial extender Beltsville Poultry Semen Extender, P2-7450, continental, Delavan, WI, USA) added at a ratio of 1:3 (semen: extender). The osmolarity of the locally prepared extender used for the cryopreservation was 320 mOsmol/kg and pH of 6.7. The diluted semen was distributed equally in 60 sterile glass tubes each. The experimental layout is presented in Table 4.

The CPAs were supplemented to each tube 1:5 v/v as a final concentration. The semen samples were equilibrated in a refrigerator at 5°C for 40 minutes (Silyukova et al., 2022). Equilibration is a process that helps the spermatozoa become more resistant to the effects of cold shock. During equilibration, the spermatozoa are permeated with glycerol, which allows for the establishment of ionic and osmotic equilibrium with the surrounding media. This equilibrium helps protect the spermatozoa during the cryopreservation process. The freezing procedure was followed by the static vapor freezing method. Sample freezing started by placing the tubes on racks in a grill wide-mouthed liquid nitrogen container kept 32 cm away from the brim (mouth) of the container. After vapor freezing, the straws were collected and plunged into pre-cooled goblets for storage. For the post-thaw evaluation, the sperm samples were thawed after 7 days by keeping the sperm in the air for 90 seconds and then in the water bath of 37°C for another 60 seconds. The mass sperm motility, progressive sperm motility, morphological abnormalities, *in vitro* viability, and acrosome integrity of the frozen semen were evaluated following Gerzilov (2010).

Table 4. Experimental groups for post-thaw sperm quality analysis

Treatment	Type of extender	Number of replications
E1	Semen diluted with homemade extender	20
E2	Semen Diluted with DMF extender	20
E3	Semen diluted with Beltsville PSE	20

E: Extender, DMF: Dimethyl-formamide, PSE: Poultry semen extender

Post-thaw semen quality assays

Semen was evaluated based on ejaculate volume, color, and concentration using a standard hemocytometer, motility, viability, and morphology percentage of spermatozoa. Mass and progressive sperm motility were assessed microscopically (400×) by putting a drop of semen on the slide. For morphological evaluation, semen was mixed with 1.6% eosin and 6% nigrosine and observed under a light microscope (×1000 magnification, MSC-P200, China) under oil immersion. A total of 200 spermatozoa were examined to determine the percentage of abnormal sperm using the Eosin-nigrosine stain. The stain was applied at a magnification of 1000X to assess the *in vitro* viability of the spermatozoa (Zong et al., 2022).

Artificial insemination and fertility evaluation

For this purpose, a total of 160 adult hens with similar age groups (35-40 weeks of age) were used. Forty hens were used in each treatment (Table 5). The hens were divided into four pens, each pen containing 10 hens, and kept for 20 days without exposure to males. Each extender and fresh semen were inseminated for fertility evaluation. The insemination was performed during the afternoon since during the morning, most hens have an egg in their oviducts, thus obstructing the free passage of semen to the ovary. A volume of 0.3 ml of thawed semen was inseminated at a 7-day interval over three weeks. The vaginal artificial insemination was performed using a 1 ml capacity sterile syringe (Getachew et al., 2015). A total of 400 eggs, 100 eggs from each treatment were collected to analyze fertility in the current study. Hens in each treatment were divided into three pens containing 40 hens each as replications. Finally, 25 eggs were collected from each pen. Hatched eggs were collected in the morning. Then, uncracked and clean eggs of at least 50 g were marked and identified by pen number and treatment number, stored sharp point of the egg downward, and pre-heated for 12 hours at 25^{o}C prior to incubation. The eggs were placed randomly in racks and trays, specifically in 150-egg capacity trays at the ELERE farms hatchery unit. These trays were then subjected to incubation for a period of 18 days at a temperature of 37.5°C and a relative humidity of 60-70%. To ensure uniform development, the eggs were turned every hour at 90^{o} during the incubation period. All eggs were candled individually on day 18 of incubation. Clear eggs were removed, opened, and inspected for evidence of embryo development. In the absence of an embryo, eggs were classified as infertile. Fertility and hatchability were calculated according to the following formula.

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$$\text{Fertility (\%)} = \frac{\text{Number of eggs fertile}}{\text{Number of eggs set}} \times 100$$

$$\text{Hatchability (\%)} = \frac{\text{Number of eggs hatched}}{\text{Number of Eggs fertile}} \times 100$$

Table 5. Experimental groups for fertility and hatchability analysis

Treatment	Type of extender	Number of hens	Number of pens	Number of hens in each pen	Number of eggs collected for incubation
E1	Semen stored using a homemade extender	40	4	10	100
E2	Semen stored using DMF extender	40	4	10	100
E3	Semen stored using Beltsville PSE	40	4	10	100
E4	Fresh semen (control)	40	4	10	100

E: Extender, DMF: Dimethyl-formamide, PSE: Poultry semen extender

Statistical analysis

The data collected during the study period were subjected to Analysis of Variance (ANOVA) using the latest version of STATA, version 12. The data on semen quality parameters were analyzed using ANOVA. When F-test is found significant, means were compared using LSD. The p-value less than 0.05 was considered to determine a statistically significant difference ($p < 0.05$). A one-way completely randomized design was utilized to evaluate the effect of CPAs on sperm quality assays.

RESULTS

Fresh semen characteristics

A summary of the results of semen characteristics addressed in this study is presented in Table 6.

Table 6. General semen characteristics of the Horro chicken breed

Semen characteristics	Mean semen characteristics
Ejaculate volume (ml)	0.36
Color	Milky white
Texture	Moderate viscous
Sperm total concentration/ml	$5.5X10^9$
Sperm count/ejaculate	$1.98X10^9$
Ph	7.2

Effect of cryoprotectants on sperm quality

The effect of CPAs on the sperm quality of Horro chicken breed is presented in Table 7. There were no significant differences in mass motility across CPAs ($p > 0.05$). However, there were significant differences between homemade and commercial extenders regarding progressive sperm motility, motility, and *in vitro* viability ($p < 0.05$). Regarding the commercial extender, there were no significant differences in all sperm quality parameters ($p < 0.05$). The semen preserved using commercial extenders indicated a significantly higher morphologically normal sperm and *in vitro* viability rate, compared to the homemade extender ($p < 0.05$). Percent progressive motility recorded the same for Dimethyl-formamide and Beltsville Poultry semen extender.

Effect of cryoprotectants on fertility and hatchability

Fertility and hatchability data are presented in Table 8. There were no significant differences across all extenders in terms of fertility and hatchability rate ($p > 0.05$). However, a significant difference was observed between the fresh semen inseminated (control treatment) and cryopreserved semen using extenders ($p < 0.05$).

Table 7. Effect cryoprotectants on post-thaw sperm quality of Horro breed

Mean ± SE Sperm Parameters / Factors	Mass motility (%)	Progressive motility (%)	Morphologically normal (%)	Viability (%)
Homemade extender	48.5 ± 1.5^a	23.75 ± 0.81^b	55.25 ± 1.11^b	33.2 ± 0.96^b
DMF	51 ± 1.52^a	28.2 ± 0.56^a	64.25 ± 0.91^a	42.75 ± 0.73^a
Beltsville PSE	51.5 ± 1.31^a	28.2 ± 0.57^a	62.05 ± 0.70^a	42.15 ± 0.52^a

SE: Standard error, DMF: Dimethyl-formamide, PSE: Poultry semen extender, ^{ab}: Different superscript letters within the same column show significant differences among the groups ($p < 0.05$).

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Table 8. Effect of cryoprotectants on fertility and hatchability of Horro breed sperm

Factor \ Treatment	Homemade extender	DMF	Beltsville PSE	Control (Fresh semen inseminated)
Fertility rate (%)	41 ± 1.82[b]	48 ± 2.27[b]	46.25 ± 1.31[b]	87.93 ± 1.64[a]
Hatchability rate (%)	77.23 ± 2.25[b]	80.25 ± 1.31[b]	78 ± 2.68[b]	87.25 ± 1.03[a]

DMF: Dimethyl-formamide, PSE: Poultry semen extender, [ab]: Different superscript letters within the same row show significant differences among the groups ($p < 0.05$).

DISCUSSION

Semen color depend on the species of roosters used. However, the milky white color of the semen observed in this current study is consistent with previous reports (Hafez and Hafez, 2000; Peters et al., 2008; Mussa et al., 2023). The color of domestic fowl semen varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands, from a relatively high sperm density or degrees of clear to milky white, with increased sperm numbers (Hafez and Hafez, 2000)**.** According to Gordon (2005), the average ejaculate volume of semen using the abdominal massage technique was 0.25 ml. Bah et al. (2001) also reported an ejaculate volume of 0.28 ml in Nigerian local cocks. Cole and Cupps (1977) reported ejaculate volume within the range of 0.1-1.5 ml per ejaculation using abdominal massage techniques. Moreover, Hafez and Hafez (2000) indicated that the average sperm volume collected from white leghorn varied from 0.2 to 0.5 ml. These studies are in agreement with the result found in the current study which was 0.36 ml/ejaculate.

The average sperm concentration in the present study was $5.5X10^9$ ml (Table 6). Results of studies performed by David et al. (2015) and AL-Saeedi et al. (2019) indicated the concentration of sperm ranging $3.4\text{-}6.8 \times 10^9$ ml. According to Gordon (2005), the average sperm concentration is 5000 × 106 sperm/ml. The sperm concentration recorded in the present study is within the range reported by Hafez and Hafez (2000), that is $3000\text{-}7000\times10^6$ spermatozoa/ml. The sperm concentration is attributed to the alkaline nature of the accessory sex gland fluid, as reported by Bah et al. (2001) and Peters et al. (2008). The use of preserved semen in poultry production is markedly less than in mammals due to the low resistance of poultry spermatozoa to heat shock, leading to a reduction of the fertility of thawed semen (Andreea and Stela, 2010; Partyka and Niżański, 2022). Poultry sperm are more susceptible to damage caused by extreme heat changes compared to mammalian sperm. This increased sensitivity is attributed to the higher levels of polyunsaturated fatty acids present in poultry sperm (Bréque et al., 2003). Despite the implementation of various protocols in cryopreservation to prevent damage to sperm, the viability of post-thaw sperm is still not satisfactory (Bacon et al., 1986; Gliozzi et al., 2011). Therefore, it is recommended to develop strategies that can reduce these structural and biochemical damages.

An evaluation of the extenders on quality of cryopreserved Ethiopian indigenous chicken semen showed that Dimethyl Formamide extender yielded a higher progressive motility percentage (28.2 ± 0.56), *in vitro* viability percentage (42.75 ± 0.73), and morphologically normal sperm percentage (64.25 ± 0.91), compared to other treatments ($p < 0.05$, Table 3). These results were similar to those reported by Łukaszewicz et al. (2008), indicating that the egg yolk and sodium citrate extender developed by Lukaszewicz (2002) yielded better results in gander semen. This result is supported by the report of Christensen (1995) in which the sperm quality attributes are highly affected by CPAs and osmolarity.

CONCLUSION

Post-thaw quality of sperm is highly dependent on the use of appropriate semen extender and freezing procedures. In this study, Dimethyl-formamide extender demonstrated better results in all sperm quality parameters, except for mass motility, when compared to the homemade extender and commercial Beltsville PSE. However, it is important to note that the overall sperm quality and fertility outcomes were still lower compared to those observed in mammalian species. Further studies are recommended to develop standard freezing procedures and the use of cryoprotectants. Based on the results obtained regarding sperm quality and fertility rates, it is concluded that poultry semen cryopreservation is more suitable for establishing a semen biobank rather than for commercial use. However, further studies are needed to identify more effective procedures and cryoprotectants that can enhance post-thaw sperm quality even further.

DECLARATIONS

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Authors' contributions

Tarekegn Getachew, Gebeyehu Goshu and Alemayehu Lemma designed the experiments and Tarekegn Getachew performed the experiments. Tarekegn Getachew derived the models and analyzed the data. Gebeyehu Goshu and Alemayehu Lemma assisted with standardizing data collection and data analysis. Tarekegn Getachew wrote the manuscript in consultation with Gebeyehu Goshu and Alemayehu Lemma. All authors read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have declared that no competing interest exists

Ethical consideration

All ethical issues, including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, have been checked by all authors.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author (T. Getachew), upon reasonable request.

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Pathologic-anatomical Changes in the Comorbidity of Eimeriosis and Tuberculosis in Domestic Chickens and Decorative Pheasants (*Phasianus colchicus* L., 1758)

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ABSTRACT

The study of patho-anatomical changes is essential in identifying pathological processes and diagnosing especially comorbid pathologies. The aim of this study was to reveal pathological changes and differences in the spontaneous comorbidity of tuberculosis (*Mycobacterium avium*) and eimeriosis (*Eimeria* spp.) in adult chickens and ornamental pheasants. The paper highlighted the results of pathological-anatomical changes in adult domestic chickens (n = 17) and ornamental pheasants (n = 5) with spontaneous comorbidity of eimeriosis and tuberculosis. Mycobacteria was detected using bacterioscopy of smears-prints from fragments of organs and *Eimeria* oocysts were detected by the Füllenborn flotation method. In pheasants, tubercular granulomas were found in the intestine, liver, and spleen in combination with scarring and swelling of the wall and mesentery, and venous stasis in the mesenteric vessels. In addition, hematomas and organ destruction in the liver and spleen were found in pheasants. In domestic chickens, tuberculous granulomas and steatosis were found in the liver. In the intestines, there were indications of mucocatarrhal inflammation, edema, hyperplasia, and small hemorrhages in the area of the cecal-intestinal diverticulum. Tuberculous nodules, internal hemorrhages around the perimeter of the tubercle, and devascularization were observed in the spleen. The present study revealed notable differences in the pathological and anatomical changes resulting from the comorbidity of eimeriosis and tuberculosis in domestic chickens and pheasants.

Keywords: Avian tuberculosis, Comorbidity, Eimeriosis, Intestine, Liver, Pathological change, Spleen

INTRODUCTION

In Ukraine, around 50% of chickens are raised in individual auxiliary farms. These farms commonly employ extensive rearing systems and walking areas for joint maintenance of different species and age groups of poultry, which increases the risks of the occurrence and spread of infectious diseases (Liulin, 2023). The existing variability of pathogens and the associative course of diseases or pathologies (comorbidity), especially with a weakened immune response of the body, create certain difficulties in their diagnosis (Campbell-Scherer, 2010; Shin and Shin, 2021). Avian eimeriosis remains a serious problem in poultry production (Dalloul and Lillehoj, 2006) and requires continuous control (Quiroz-Castañeda and Dantán-González, 2015) due to a high prevalence of up to 100% and a mortality of up to 70% in the absence of treatment. Poultry tuberculosis, caused by the bacterium (*Mycobacterium avium*), has a significant impact not only on chickens but also on domestic animals (Polaček and Aleksić-Kovačević, 2016; Komatsu et al., 2017; Lamuka et al., 2018) since the causative agent can induce relevant pathologies in a wide range of species. Moreover, this pathogen poses a serious underestimated threat to human health, especially in an immunodeficient state (Azar et al., 2019; Zhurylo et al., 2020; Kaczmarkowska et al., 2022). Moreover, it causes significant sanitary-epidemiological and social problems due to the possibility of transmission of the causative agent of avian tuberculosis from domestic and ornamental birds to humans (Slany et al., 2016; Patiño et al., 2018; Busatto et al., 2019).

Avian tuberculosis often remains undetected during a person's lifetime, as it typically manifests without noticeable clinical symptoms. It is commonly diagnosed only posthumously, highlighting the challenge of identifying the infection while the individual is alive (Crilly et al., 2021). In cases of avian tuberculosis, the primary diagnostic indicators are the distinctive patho-anatomical changes observed. Unlike mammals, birds primarily develop the tuberculosis complex (the focus of typical pathological and anatomical changes) in the intestine and liver when infected through the alimentary route. In the case of a generalized form of the infection, it can also affect other organs, including the spleen, exhibiting characteristic pathological and anatomical alterations. In this case, granulomas specific to tuberculosis are detected in the organs, the

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intensity of which depends on the level of natural resistance and the ability of the organism to regenerate, which affects the development and prognosis of the disease (Álvarez et al., 2017; Al-hamadawi et al., 2017; Zikovitz et al., 2018). One of the models of comorbidity of tuberculosis in poultry from farms unfavorable for this disease can be its combination with eimeriosis. As noted, mycobacteria can penetrate, survive and persist in the body of protozoa, including *Eimeria*, contributing to the potential simultaneous infection of birds with *Eimeria spp.* and *Mycobacterium* (*M*) *avium* complex (Lande, 2019; Butler et al., 2020). In addition, both diseases (tuberculosis and eimeriosis) have similar clinical symptoms, including general weakness, cyanosis, diarrhea, cachexia, reduction in egg production, and even cessation of egg-laying in sick chickens.

The pathogenic immunosuppressive effect of *Eimeria* on the body of sick chickens and the phenomenon of tropism of *mycobacteria* to immune structures and their ability to cause an immunodeficiency state contribute to the specified comorbidity, which has an applied diagnostic value (Kim et al., 2019; Ioakeim-Skoufa et al., 2020; Zhurylo et al., 2020). During the patho-anatomical diagnosis of eimeriosis and/or tuberculosis in poultry, it is advisable to consider the organs' topographic and morphological features and the presence of lymphoid elements. The localization and nature of lesions of these structures may differ both within the species and in different age groups of domestic, decorative, or wild birds. Moreover, the scale of indicators of patho-anatomical changes in avian tuberculosis needs to be supplemented with new data, which are the results of complex multi-disciplinary studies, and contribute to the improvement of its diagnosis (Özen et al., 2016; Mabelebele et al., 2017).

Thus, in peacocks, morphological changes caused by the causative agent of avian tuberculosis are found in the glandular stomach, which is rich in lymphoid structures (Ciobotaru et al., 2012; Mayahi et al., 2013; Liakhovich and Maslak, 2021). In pheasants, the saturation of the intestinal wall with lymphoid elements leads to the manifestation of specific pathological anatomical changes during the development of tuberculosis in its structures (Kovtun and Harchenko, 2005; Parisa et al., 2019). This determines the high specific sensitivity of pheasants to the causative agent of tuberculosis and, in epizootic centers, threatens their extinction (Álvarez et al., 2017; Lyakhovich et al., 2019). The level of susceptibility of domesticated pheasants to tuberculosis is also affected by their long-term captivity and insufficient solar insolation (Bekele et al., 2018). Vitamin D deficiency contributes to the development of active tuberculosis infection (Babazadeh et al., 2022). The phenomenon of immunosuppression, both for eimeriosis and tuberculosis, provokes manifestations of comorbidity with other diseases or pathologies (Babu and Nutman, 2016; Olmedo-Reneaum et al., 2020).

In the spectrum of liver pathologies of various human genesis, patterns of comorbidity of tuberculosis-protozoan lesions were recorded (Tsyrkunov and Prokopchik, 2018). When typical pathological changes associated with tuberculosis are identified in poultry, it is crucial not to disregard the possibility of a concurrent occurrence of tuberculosis with diseases that resemble it in terms of pathogen localization and/or lesion manifestation. The diagnosis of eimeriosis-tuberculosis comorbidity in poultry and the peculiarities of its patho-anatomical manifestation will expand the informative scale of pathogenetic links and diagnostic indicators of these diseases.

The purpose of the current study was to investigate and classify macroscopic changes in the liver, spleen, and intestines of domestic chickens and decorative pheasants with comorbidities of tuberculosis and eimeriosis.

MATERIALS AND METHODS

Ethical approval

These studies were conducted in compliance with the ethical norms and principles of the requirements of the European Union.

Pathological and anatomical analysis

A total of 22 dead birds were subjected to a comprehensive pathological and anatomical examination. The sample consisted of 17 chickens, including 7 female Rhode Island breed, 5 female Leghorn breed, and 5 roosters of the *Adler silver* breed aged between 2 and 3 years. Additionally, the study involved 5 adult ornamental pheasants, comprising 3 males aged 2.5, 4.5, and 6 years, as well as 2 females aged 1.5 and 2.5 years. Autopsies of bird carcasses were performed at Department of Normal and Pathological Morphology of the Kharkiv State Zooveterinary Academy, Ukraine (now the State Biotechnological University, Ukraine) by eviscerating the supine position (Dobin and Kokurichev, 1963), following the method of anatomical preparation of the liver, spleen, intestinal tube and its mesentery. To ensure proper preservation, the corpses of the deceased birds were transported from the auxiliary farms and the ecopark of the Kharkiv region in special thermal containers with ice, maintaining a temperature of +4°C within 12 hours after the birds' death.

Coproscopic analysis

A small amount (the size of a pea) of the permeable intestine (chyme) from different parts of the intestine was carefully mixed with 1 ml of 50% water-glycerol solution in a chemical beaker. Then, a drop of this solution was applied to a glass slide, covered with a coverslip, and subjected to microscopy (magnification ×80 and ×400) to identify *Eimeria* oocysts and their development stages.

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Flotation method

Eimeria oocysts are detected by the Füllenborn flotation method. To do this, samples of feces (3 g) were taken from the end part of the intestine (cloaca) of bird corpses, which were mixed with 30 ml of a saturated NaCl solution and filtered through a metal filter (hole size 0.8 - 1.0 mm). The filtrate was settled for 30 minutes, after which a metal loop (diameter 0.8 cm) was used to take samples from the liquid surface of the studied samples. Specifically, 3 drops of the surface film were collected and transferred to a glass slide and microscopically examined for the presence of *Eimeria* oocysts. The number of oocysts in 1 gram of feces was determined using Goryaev's kamera (Halat et al., 1999). The species affiliation of pathogens of *Eimeria spp.* was determined based on morphological examinations, including the analysis of oocyst shell shape, color, presence or absence of micropyle, polar granules, and residual body in both oocysts and sporocysts. Additionally, their biological characteristics such as sporulation periods, length of prepatent and patent periods were considered. The obtained results were then compared with the data from determination tables, specifically those outlined by Pellerdy (1974).

Microbiological analysis

Mycobacteria were detected by bacterioscopy of smears-prints from fragments of organs affected by granulomas: liver, intestinal wall, and spleen according to the Ziel-Nielsen method (Asmolov, 2002).

Statistical analysis

The obtained results were analyzed using Statistica 10 software (StatSoft Inc., USA). Differences between the parameters of the experimental groups were determined using ANOVA. The test Duncan was used to determine the p value. The results were presented as the mean ± standard error of the mean (M ± SE). The difference between groups was considered significant at $p < 0.05$.

RESULTS

Based on patho-anatomical changes and the results of microscopy of samples of domestic chickens and decorative pheasants, the comorbidity of eimeriosis and tuberculosis was established. The causative agents were identified as *Eimeria*. Bacterioscopy of smears-imprints taken from affected areas of the liver, intestinal tube walls, and spleen was performed. The smears were stained using the Ziel-Nielsen method. The examination revealed the presence of red acid-resistant mycobacteria, which appeared as rod-shaped bacteria either singly or in pairs.

The pathological examination of pheasant corpses

The carcasses of the investigated male pheasants were, to varying degrees, exhausted (with skeletal muscle atrophy), dehydrated, and with signs of general anemia. The carcasses of female pheasants were moderately fat with anemia. Macroscopically, pheasants showed changes in the liver, spleen, intestinal tube, and mesentery.

The intestinal tube was deformed to varying degrees due to asymmetrically located spherical thickenings in the form of granulomas of different diameters and degrees of maturity, which were visible from the side of the highly moist and shiny serous membrane. In male pheasants, the sections of the intestinal wall between the granulomas were wrinkled. The intestinal tube was slightly shortened (without characteristic anatomical bends, Figure 1). The lumen of the intestinal tube was empty. On a cross-section in most areas, it was narrowed and deformed due to transmural granulomas, and individual post-traumatic ulcers with diffuse hemorrhages (after the destruction of granulomas) were observed. Blood stagnation in the mesenteric venous vessels was observed. The blood was a dark cherry-colored, asphyxial type (lightens on contact with air).

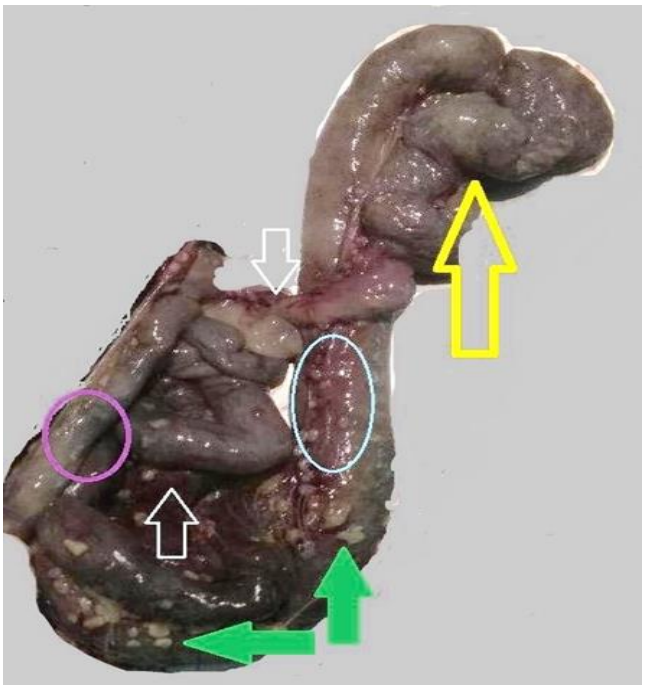

Figure 1. Macroscopic intestinal lesions of a decorative pheasant with comorbidity of eimeriosis and tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2019. **Yellow arrow**: The deformation of the wall, **Green arrows**: Multiple light yellowish granulomas, **Purple oval**: The increased gloss of the serous membrane due to edema, **Blue oval**: Edema of the mesentery, **White arrows**: venous stasis in mesenteric vessels

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In female pheasants, with the use of low-multiplier optical lenses, isolated small whitish nodules were found on the side of the serous membrane of the jejunum; on the mucous membrane in different parts of the intestinal tube - partial redness, swelling, individual small hemorrhages according to the type of ecchymoses. Male pheasants were diagnosed with total destructive tuberculous jejuno-ileo-typhlitis (inflammation of the walls of the jejunum, ileum, and cecum). In female pheasants, local granulomatous tuberculous jejunitis combined with edematous-hemorrhagic enteritis was classified.

Multiple granulomas of different diameters and degrees of maturity were found in the liver of male pheasants, including one with a central area of caseous necrosis (Figure 2). Signs of tuberculous hepatitis and atrophy of the organ were observed in the liver of female pheasants (sharp edges, wrinkling of the capsule and light pink-gray color in the areas between yellowish granulomas of different diameters, weak vascularization, Figure 3). The spleen of female pheasants was slightly enlarged due to the presence of small nodules of light gray color (Figure 4, classified productive miliary splenitis).

In male pheasants, the spleen was difficult to differentiate due to extreme deformation and destruction. It revealed confluent large (0.5-0.7 cm) gray-white nodules with caseous content and spherical-ellipsoid formations of dark brown-cherry color (0.5-0.8 cm) with an area of damage up to 30-45% ($p < 0.05$) of the surface (with decay masses soaked in dark cherry blood). Tuberculous granulomas and subcapsular hematomas were detected. Coproscopic examinations conducted on samples obtained from the intestinal tubes of the examined pheasant carcasses revealed a 100% infestation rate by *Eimeria*. The intensity of invasion was 46.7 ± 3.9 - 85.3 ± 6.4 oocysts per gram of feces by the species: Eimeria colchici (Norton, 1976), *Eimeria phasiani* (Tyzzer, 1929) and *Eimeria duodenalis* (Norton, 1976), respectively 24, 7%. 63.4% and 11.9% of the total number of oocysts ($p < 0.05$).

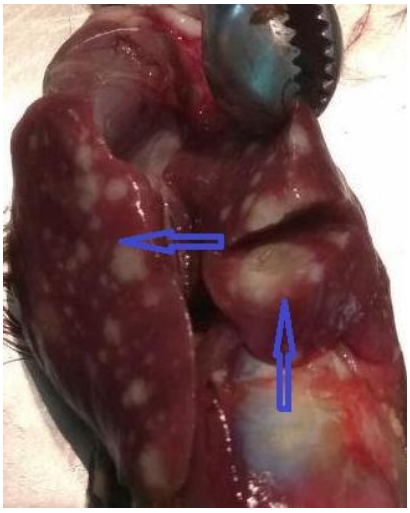

Figure 2. Macroscopic lesions of the liver of a decorative pheasant with comorbidity of eimeriosis and tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2019. Multiple miliary and mature large focal granulomas (arrows).

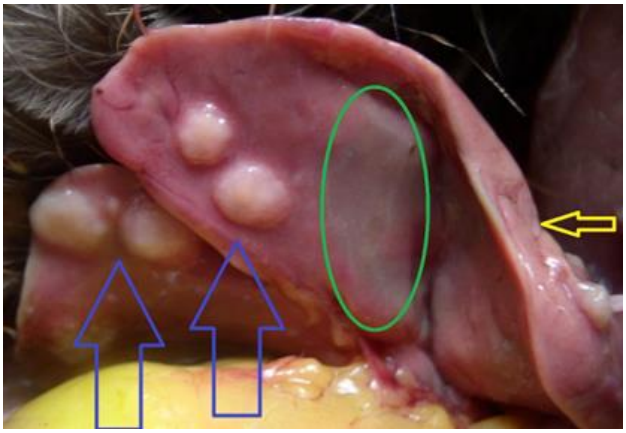

Figure 3. Macroscopic lesions of the liver of a female pheasant with comorbidities of eimeriosis and tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2019. The isolated large focal granulomas (**blue vertical arrow**); signs of atrophy (too sharp edges of the organ and deformed surface, **yellow arrow**); devascularization of the liver (presence of bloodless grayish areas, **green oval**).

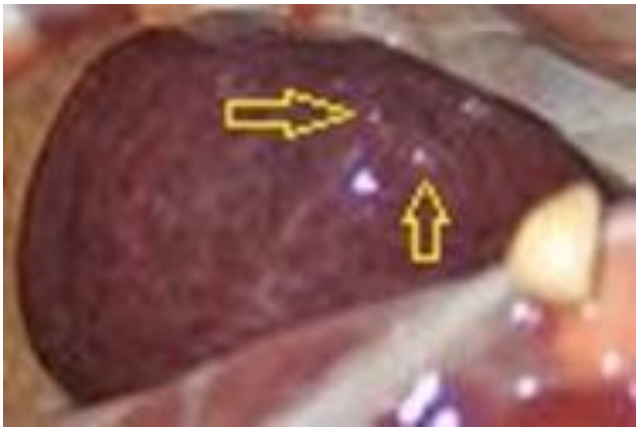

Figure 4. Spleen of a pheasant with concomitant diseases of eimeriosis and tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2019. An increase in the volume of the organ due to small light gray granulomas (**yellow arrows**).

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Results of pathological examination of chicken carcasses

According to the results of the pathological examination of the carcasses of domestic chickens (12 females and 5 roosters), the poultry had different levels of fatness. During the examination of the organs in the thoracic and abdominal cavity of chickens, typical changes indicative of tuberculosis were observed. These changes manifested as specific granulomas present in the liver, intestinal wall, and its mesentery. At the same time, in three out of seven chickens, multiple tubercular nodules in the liver were combined with individual specific miliary granulomas in the intestinal wall (their visualization was facilitated by the use of low-optic lenses). Destructive granulomatous miliary, small-focal, and large-focal hepatitis with the destruction of the capsule and parts of the organ was diagnosed in the liver of the examined chickens (Figure 5).

In the first group of chickens, the livers of five exhibited fragility upon removal from the thoracic cavity, lacking clear differentiation of the capsule. These livers displayed multiple specific tuberculous nodules of varying diameters, characterized by a light-yellowish color. These nodules were observed as miliary and small foci. Additionally, there were indications of steatosis, with the organ appearing enlarged due to capsule rupture. The color of the liver exhibited variation, with light beige areas between the nodules and pale yellowish nodules themselves. During the liver sectioning process using a scalpel, fatty deposits were left on the blade, and light yellowish caseous formations were discovered within the organ (Figure 6).

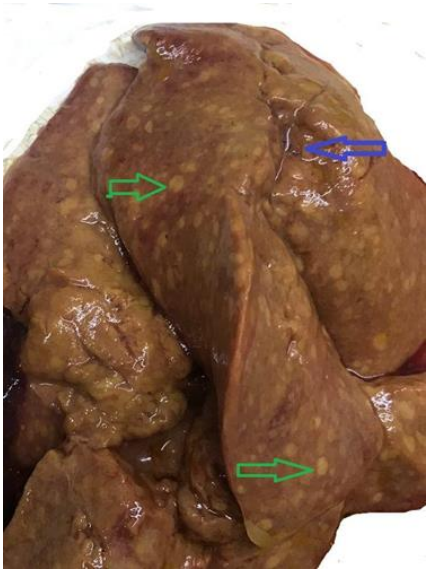

Figure 5. Macroscopic lesion of the liver of a domestic chicken (Leghorn breed) eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Multiple miliary and small-focal granulomas of light yellowish color under the capsule (**green arrows**) and in the thickness of the organ (**blue arrow**). Kharkiv region, Ukraine, 2020.

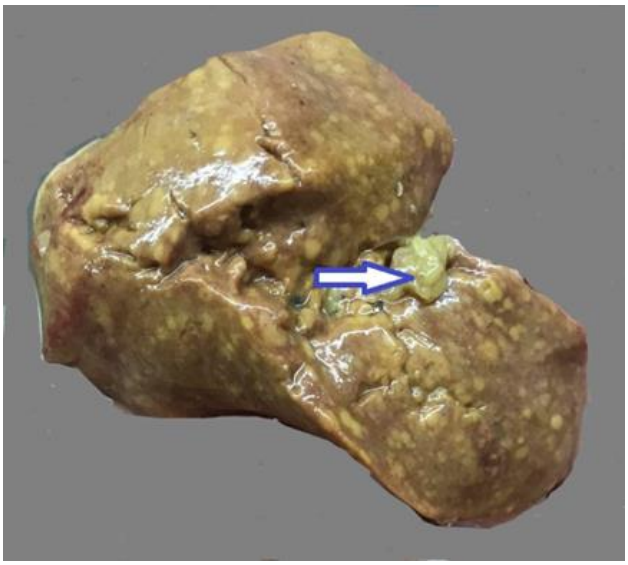

Figure 6. A fragment of the liver of a domestic chicken (breed - Leghorn, age 2.5 years) for comorbidities of *Eimeria* and tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Macroscopic lesions of the liver: Multiple granulomas of pale-yellow color, rupture of the capsule and parenchyma of the organ, caseous substrate in the area of the rupture (**arrow**).

Coproscopic examinations of the intestinal tube material from the chicken carcasses revealed the presence and identification of various *Eimeria* species. These included *Eimeria acervulina* (Tyzzer, 1929), *Eimeria brunetti* (Levine, 1939), *Eimeria maxima* (Tyzzer, 1929), *Eimeria mitis* (Tyzzer, 1929), *Eimeria necatrix* (Jonson, 1927), *Eimeria praecox* (Jonson, 1927), and *Eimeria tenella* (Raillet and Lucet, 1891). These species accounted for 18.6%, 7.6%, 16.5%, 4.9%, 11.3%, 2.4%, and 38.7% ($p < 0.05$) of the total number of oocysts, respectively. The intensity of invasion ranged from 140.6 ± 4.8 to 263.2 ± 5.7 oocysts per gram of feces.

With the help of low-optical lenses, single whitish miliary granulomas with subserosal localization were observed in the intestinal wall of three chickens (within the topographic boundaries of the jejunum, Figure 7). In addition to the mentioned changes, some miliary and small nodular tuberculous nodular lesions of the intestinal mesentery, had elongated ellipsoid shape and pale pink shade (Figure 8). In individuals of roosters and chickens, diffuse hemorrhages were detected from the side of the serous membrane in the intestinal wall, particularly the duodenum, due to the use of low-optical lenses (Figure 9).

Significant changes were observed in the caecum of the examined domestic roosters. These changes included the presence of stagnation, accumulation of exudate masses, detritus, and gases. As a result, a local flask-like expansion and stretching of the intestinal wall occurred. In certain areas, the intestinal wall became thin to the point of translucency, which could be attributed to the desquamation of the epithelium (Figure 10). Hyperplasia of the cecal tonsils (tonsils), swelling, mass of exudate and/or decay were found in part of the studied chickens at the level of the mucous membrane

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in the area of the cecal diverticula (Figure 11). Brown-red masses of mucous-hemorrhagic exudate and disintegration of the intestinal wall were found in individual chickens in the lumen of the caecum (Figure 12).

According to the sectional examination of the intestine in chickens, lesions of two categories were established, including non-intense productive (granulomatous) subserous enteritis within the jejunum, characteristic of tuberculosis with intestinal localization of changes, and intense edematous-hemorrhagic and/or desquamative typhlitis (in some individuals) or sectoral moderate duodeno-jejuno-ileo-typhlitis (in the majority of examined chickens), typical for poultry eimeriosis.

Thus, the obtained results of studies of pathoanatomical changes in the intestine and their classification according to the comorbidity of tuberculosis/eimeriosis in chickens and pheasants can serve as diagnostic markers (Table 1). Except for one rooster, the spleen of the studied domestic chickens had a preserved shape, a moderately increased volume, and a uniform color (from light cherry to dark cherry brown in different individuals, Figure 13). An enlargement of the spleen due to hyperplasia was observed. In some individuals, the enlargement was also associated with venous stasis. Importantly, the integrity of the spleen's capsule remained preserved throughout the examination. In one examined rooster, the spleen was deformed due to the presence of multiple yellowish nodules (0.2-0.4 cm in size), moderately enlarged, unevenly colored, both from the surface and on the section (there were yellowish nodules on a devascularized light cherry background, the perimeter of some of which, with the help of low-power optical lenses, perifocal hemorrhages of bright red color were visualized, Figure 14).

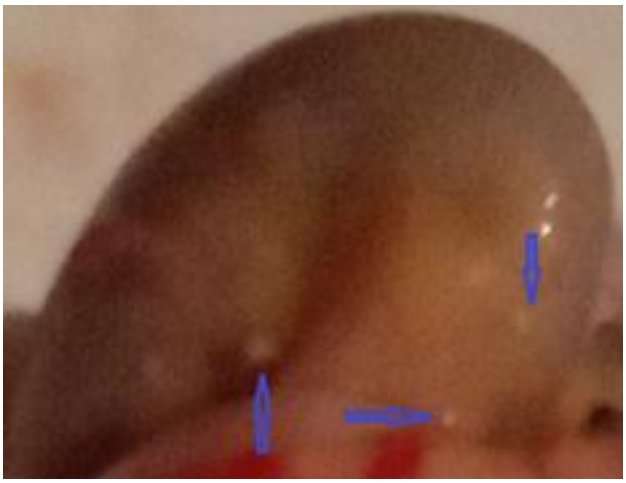

Figure 7. Macroscopic lesions of the wall of the jejunum of a domestic chicken (Leghorn breed) due to the comorbidity of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Miliary granulomas from the side of the serous membrane (**arrows**). Magnification with a small optical lens × 5.

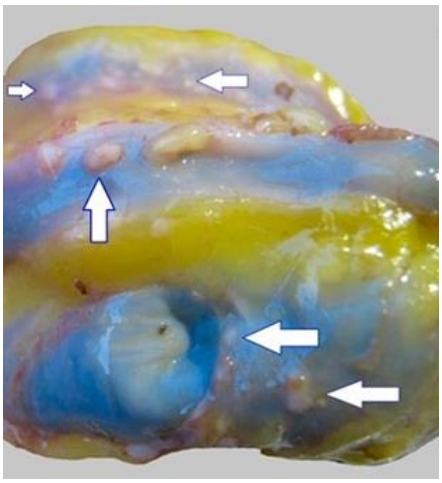

Figure 8. Lesions of the intestinal mesentery of a domestic chicken (Rhode Island breed) due to the comorbidity of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. The presence of miliary and small-focal ellipsoidal granulomas of pale pink color (**arrows**).

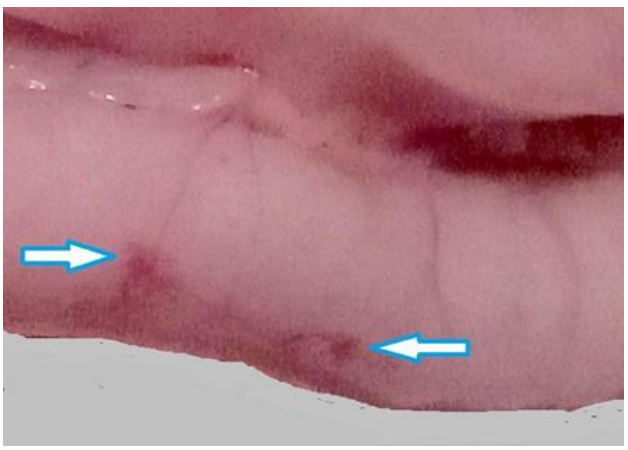

Figure 9. A fragment of the U-shaped knee of the duodenum and pancreas of a domestic rooster (*Adler silver* breed) with comorbidity of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Diffuse subserosal hemorrhages (arrow). Magnification with weak optical lens × 2.

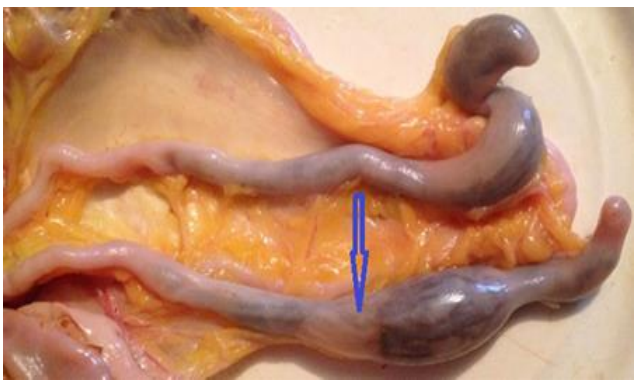

Figure 10. Caeca lesions (caeca) of a domestic rooster (*Adler silver* breed) with comorbidities of *Eimeria*/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Bulbodental expansion and thinning of the wall (arrow). Magnification with a low-optical lens × 3.

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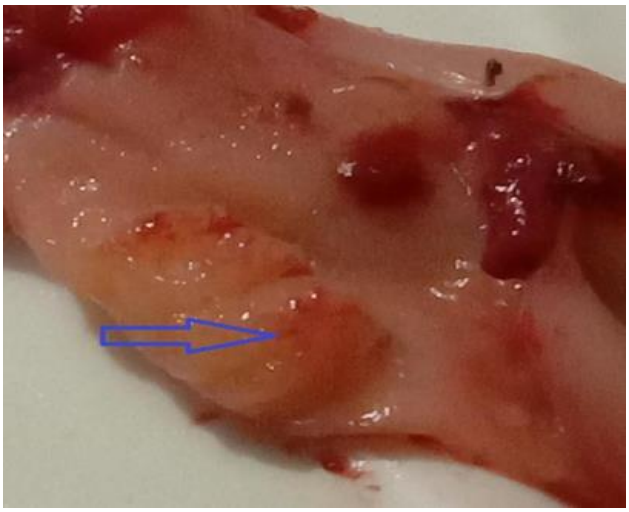

Figure 11. Cecal diverticulum of a domestic rooster (*Adler silver* breed) with comorbidity of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. hyperplasia of the lymphoid tonsils (**arrow**), swelling, and masses of exudate on the surface of the mucous membrane. Magnification with a low-optical lens × 5.

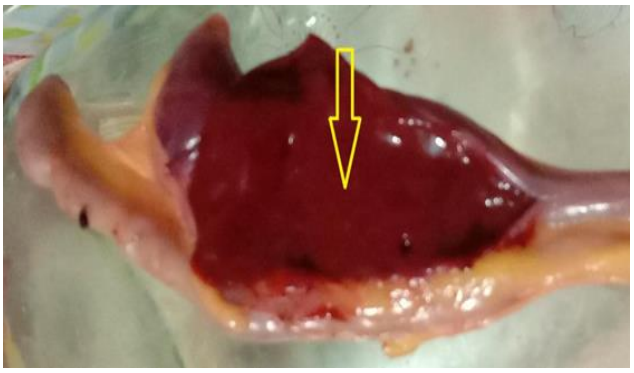

Figure 12. Damage to the caecum of a domestic chicken (Rhode Islan breed) due to comorbidity of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. The presence of masses of mucohemorrhagic exudate and decomposition products in the lumen (**yellow arrow**).

Table 1. Comparative characteristics of macroscopic indicators of intestinal lesions of domestic chickens and decorative pheasants under the comorbidity of eimeriosis /tuberculosis

Index	Domestic chickens	Decorative pheasants
Swelling of the intestinal wall	Local	Total
Hemorrhages	Diffuse subserous and small (according to the type of ecchymoses): In the serous and mucous membranes of the duodenum (due to invasion by *Eimeria acervulina*), jejunum (for *Eimeria maxima* and *Eimeria necatrix* infestations), in the mucous membrane of cecum and their diverticula (in case of *Eimeria tenella* infestation).	Diffuse post-traumatic in the area of transmural tuberculous ulcers; in the mucous membrane of the duodenum, jejunum for infestations (*Eimeria duodenalis* and *Eimeria phasiani*), caecum (*Eimeria colchici*)
Granulomas	Single, small (miliary), spherical, white, in the wall of the jejunum (visualized using low-optical lenses)	Different diameters (small and large foci) and degrees of maturity (including a light yellowish central zone of caseous necrosis) in the wall of the jejunum, ileum, and cecum
Deformation of the wall	Local, within the granulomas in the wall of the jejunum (visualized using low-optic lenses)	Total, due to granulomas and scarring (in males), local in female pheasants due to granulomas in the wall of the jejunum

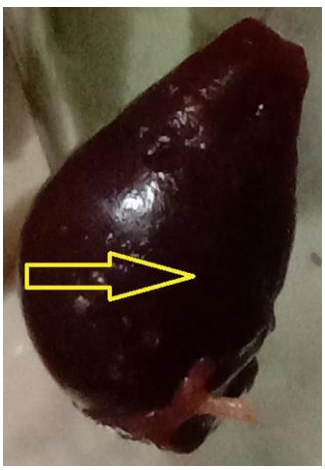

Figure 13. Spleen of a domestic cockerel (*Adler silver* breed) with comorbidities of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. The capsule tension (enlargement of the spleen); venous congestion (**yellow arrow**).

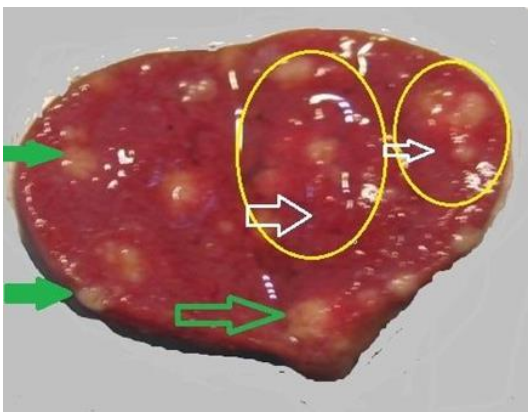

Figure 14. The spleen of a domestic cockerel (*Adler silver* breed) with comorbidities of eimeriosis/tuberculosis. The sample was collected from Kharkiv region of Ukraine, 2020. Tuberculous nodules (**green arrows**), internal hemorrhages around the perimeter of tubercular nodules (**white arrow**), white triangle in **yellow ovals**; the area of devascularization is also shown.

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DISCUSSION

In the context of comorbidity between tuberculosis and eimeriosis, the findings of the current study indicated characteristic signs of specified diseases. However, there were certain species differences between domestic chickens and ornamental pheasants. In the studied pheasants, the patho-anatomical changes were characterized by the dominance of pronounced, more intense damage to the intestinal wall with the predominance of atrophic changes in its structures, which characterized the duration of the lesions and the previous strength of the body's protective. In particular, the presence of lymphoid anatomic and morphological structures of the intestinal wall determined the peculiarities of the manifestation of pathological changes, which was also mentioned by other researchers (Kovtun and Harchenko, 2005; Parisa et al., 2019).

In lymphoid formations there were primary foci of struggle with *M. avium* were formed in the intestinal wall of pheasants (tuberculous granulomas), which appear in other organs due to the generalization of the pathogen, which is confirmed by the data (Zikovitz et al., 2018). The degree of tuberculous granulomas of the intestinal wall in pheasants differed between males and females. Thus, in male pheasants, lesions of the intestinal wall were total and transmural, which indicates their duration. In female pheasants, only isolated tubercular nodules were found on the serous membrane, and moderate superficial lesions were caused by *Eimeria spp.* (infestation intensity of 46.7 ± 3.9-85.3 ± 6.4 oocysts per gram of feces), which was consistent with a study by Goldová et al. (1998). In the intestines of the examined chickens, changes typical for inflammation were detected, in particular, damage to the surface layers of the mucous membrane (exudative with the involvement of lymphoid structures and manifestations of hyperplasia), which were caused by the action of pathogens *Eimeria spp.* (83.7 ± 11.6-161.8 ± 23.8 oocysts in 1 gram of feces), which was supported by Liulin et al. (2021), indicating similar variants of pathological changes in the intestines of pigeons with experimental eimieriosis. Kot et al. (2020) also observed signs of catarrhal-serous, catarrhal-mucous, and hemorrhagic enteritis, as well as necrosis of the structures of the intestinal mucosa, during eimeriosis in quails. The main patho-anatomical changes due to the comorbidity of tuberculosis/emmeriosis in the studied chickens were found in the liver. The degree of intestinal damage was less pronounced in chickens than in pheasants.

Thus, during spontaneous alimentary infection of pheasants with pathogens of *M. avium* and *Eimeria* spp., lymphoid components of the intestinal wall were initially damaged. The patho-anatomical changes indicated the chronic nature of pathological processes due to the possibility of remissions. At the same time, repeated cases of such infection caused total damage to the intestinal wall in pheasants, and there was a combination of signs of its tuberculous productive inflammation and atrophy due to chronic lesions and the inability to regenerate the lost structures fully. Consequently, these pathological changes resulted in spasms and wrinkling of specific segments of the intestine, attributed to scarring processes. These alterations led to narrowing, deformation, and wrinkling of the affected areas, ultimately causing the absence of chyme within the intestinal lumen. This observation aligns with the findings reported by Lyakhovich et al. (2019). The specified changes in male pheasants led to impaired function of the affected parts of the intestine, development of dehydration, general cachexia and anemia due to subcapsular hematomas of the spleen and intestinal bleeding in concomitant diseases (*Eimeria*/tuberculosis). Lyakhovich et al. (2019) indicate the differences in the manifestations of patho-anatomical changes due to tuberculous lesions in ornamental pheasants, the influence of their diet on their development, and background steatosis of the liver. The current study indicated a slightly different pattern of combined changes in the liver of chickens. Tuberculous lesions and signs of steatosis were approximately the same in severity and were easily visualized. This is explained by the higher content of lipids in the liver of domestic chickens, which in case of steatosis, contributed to the development of mycobacteria and the manifestation of the corresponding patho-anatomical tuberculosis lesions of the organ.

In case of granulomatous-necrotic tubercular hepatitis of pheasants (Curland et al., 2018), concomitant pathology (23.1%) of *Eimeria* was also detected (which was confirmed by parasitological studies) with the presence of *Eimeria* oocysts in the intestines. At the same time, granulomatous lesions of the liver were expressed to a lesser extent, compared to the changes detected in the intestine. However, it was not indicated whether the same individual pheasants simultaneously had tuberculous pathological changes and *Eimeria* invasion. *Eimeria* invasion of pheasants is quite widespread in Europe. Researchers have coproscopically detected *Eimeria spp.* (in 64% of 2-week-old pheasants and 73% of 8-week-old pheasants, Goldová et al., 2006). Gassal and Schmăschke (2006) diagnosed *Eimeria* infestation among 41% of adult pheasants with low intensity by three species, including *Eimeria phasiani*, *Eimeria duodenalis* and *Eimeria tetartooimia* (at the same time). The species *Eimeria tetartooimia* was discovered for the first time in Germany, although it is widespread in the Japanese green pheasant and is found in zoos in Japan). A total of 10 *Eimeria* species have been morphologically and molecularly identified and described in pheasants, as reported by Matsubayashi et al. (2021). Notably, three of these species, namely *Eimeria colchici*, *Eimeria duodenalis*, and *Eimeria phasiani*, have been found to have a close association with tuberculosis according to researchers from the United Kingdom. In the examined carcasses of chickens and pheasants, a simultaneous infection (comorbidity) of *Eimeria* spp. and *M. avium* was diagnosed (Norton, 1976).

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Similar pathologies in pigeons are described by Dong et al. (2018) and indicate the simultaneous detection of two to five species of *Eimeria*. The localization and degree of damage to the intestinal wall in different parts depend on the species composition (Kot et al., 2020). In particular, inflammation of the caecum caused by *Eimeria tenella* leads to their expansion, violation of evacuation ability, and stagnation of contents (chyme, inflammatory exudate, masses of detritus and gases) favors the development of pathogens in their lumen, and the creation of anaerobic conditions in particular, *Clostridium perfringens* and *M. avium* (Stanley et al., 2014; Macdonald et al. 2019; Michalska et al., 2021). This finding aligns with a recent study by Madlala et al. (2021), which demonstrated that *Eimeria* spp. parasitization disrupts the homeostasis of the intestinal microflora, leading to an imbalance. This imbalance, in turn, promotes the proliferation of clostridia bacteria. Notably, the presence of *Eimeria tenella* has been shown to facilitate the reproduction of potentially pathogenic bacteria (Chen et al., 2020). Moreover, this disruption of the intestinal microflora increases susceptibility to infectious diseases, including tuberculosis (Curland et al., 2018). The comorbidity of eimeriosis and tuberculosis in pheasants was combined with lesions of the surface layers of the intestinal wall by *Eimeria*, and its deeper layers, especially its lymphoid structures, by tuberculosis pathogens (the wall of pheasants' intestines is saturated with a significant number of lymphoid structures). When the lymphoid structures of the intestinal wall are destroyed due to infection with *M. avium* and *Eimeria spp.*, it leads to water absorption disorders, the development of severe dehydration of the body, and the death of pheasants (Yevstafieva and Kovalenko, 2019). Thus, it is appropriate to consider the caecum of pheasants as a kind of niche for the reproduction of mycobacteria. The simultaneous development of *Eimeria* in them creates prerequisites for infection, which is confirmed by Liou et al. (2001), while immunization of pheasants with a low dose of oocysts *Eimeria colchici* protects them from mass death and potentially prevents the development of anaerobic infections and tuberculosis.

The death of domestic chickens (unlike pheasants) is related to liver dysfunction, namely the development of cytolytic syndrome. A similar syndrome also leads to developing tuberculous liver damage in humans (Okusok et al., 2017). Polymorbidity is a combination of liver lesions of different genesis in humans, particularly tuberculous and protozoal (Tsyrkunov and Prokopchyk, 2018). Combined liver lesions in domestic chickens (tuberculous and steatosis) have been reported (Liakhovich et al., 2022). Miliary nodules and diffuse or petechial hemorrhages in the liver and/or spleen and intestines were found in domestic chickens that died from tuberculosis (Debelu et al., 2022).

Pathological signs of duodeno-jejuno-ileo-typhlitis were diagnosed in the intestines of most chickens in the current study. According to Yatusevich et al. (2016), the specified structural changes in the intestinal wall of chickens should be considered morphological markers of eimeriosis since certain species of its causative agents have their own species-specific localization in the intestines. During the spontaneous invasion of Japanese quails by *Eimeria tsunodai*, morphological changes were detected in the caeca. The degree of their damage increased with the age of the bird. It was characterized by the destruction of the mucous membrane of the caeca, which led to atrophy of the folds and degradation of the mucous membrane, accompanied by the development of necrotic enteritis. It was accompanied by the loss of body weight of the quails and dehydration (Gesek et al., 2015). A similar picture was also observed in the studied pheasants, which were diagnosed with dehydration and general cachexia, manifested by skeletal muscle atrophy.

Both typical tuberculous lesions and their combination with natural complications (decomposition nodes and hemorrhages/hematomas) were diagnosed in the spleens of the studied pheasants and chickens. Critical pathologies in pheasants were subcapsular hematomas of the spleen, its destruction with devascularization, and the development of regular hemorrhagic shock. Combined tuberculous lesions of the spleen and its hematomas were dominant in the studied pheasants. The indicated massive damage to the spleen together with intestinal pathology played a decisive role in the death of the pheasants. In general, pathological changes in organs (liver, intestines, and spleen) were important criteria for diagnosing comorbidity of tuberculosis and eimeriosis in poultry. In pheasants, the nature of the detected changes in visceral organs was characterized by the dominance of specific tuberculous changes in the intestines and spleen, while in chickens, changes in the spleen were recorded in 5.8% and were not as critical as in pheasants. Liver failure was dominant in the mechanism of death of chickens. Manifestation of pronounced tuberculous pathological changes in the liver of female chickens was facilitated by its background damage - fatty hepatosis provoked by a high-energy diet. Signs of steatosis were clearly visualized, which affected its color in the areas located between specific tubercular nodules. It is known that the color of the liver in birds depends, in particular, on the accumulation of lipids that enter the body with feed and as a result, give the organ a yellowish tint (Al-hamadawi et al., 2017).

In the studies of domestic chickens, the background color of the liver was light beige precisely because of excessive lipid infiltration, which is consistent with other studies (Kälsch et al. 2011; Zaefarian et al. 2019). The results of the postmortem examination of chickens and pheasants that died due to comorbidities of *Eimeria*/Tuberculosis were determined. Among decorative pheasants, age is considered a significant factor as the risk of infection by potential pathogens tends to increase with age. On the other hand, in domestic chickens, the risk of liver damage caused by *Eimeria* and tuberculosis pathogens is heightened due to the earlier onset of steatosis, which is attributed to a high-energy diet.

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CONCLUSION

The results revealed significant differences in pathological changes in pheasants and domestic chickens affected by the comorbidity of eimeriosis and tuberculosis. In pheasants, results indicated intestinal damage, tuberculous oviductal ileotyphitis, edematous-hemorrhagic enteritis, cicatricial narrowing, and deformations. Signs of atrophy, miliary, and isolated large-focal tuberculous granulomas were observed in the liver. The spleen demonstrated tuberculous granulomas and subcapsular hematomas. In domestic chickens, the primary lesion was localized in the liver, characterized by destructive granulomatous miliary, small-focal and large-focal hepatitis, accompanied by steatosis and damage to the capsule and parenchyma of the organ. The intestine showed local single miliary tuberculous granulomas and changes of *Eimeria* genesis, mucous-catarrhal enteritis, edema, hyperplasia with small hemorrhages. In the spleen, tuberculous nodules with peripheral hemorrhages and devascularization were found. These findings highlight the need for further research on the pathogenesis and pathoanatomical pattern of comorbidity and polymorbidity of infectious and invasive diseases of domestic and exotic birds.

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Availability of data and materials

All data from the current study is available by request.

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Author's contributions

Petro Lulin conducted a coproscopic study and described its results. Lyubov Lyakhovych conducted the pathological examination, took photographs, described and analyzed the data. Mykola Bogach conducted and described the morphometry of eimeriosis pathogens. Anastasia Ulyanytska participated in patho-anatomical autopsies, conducted microbiological studies, bacterioscopy, and identification of the causative agent. Petro Lyulin, Lyubov Lyakhovych and Mykola Bogach wrote the manuscript. All authors reviewed and approved the final version of the manuscript for publication in this journal.

Competing interests

There are no stated conflicts of interest by the authors.

Ethical considerations

All authors reviewed the manuscript for ethical issues such as plagiarism, consent to publish, misconduct, forgery and/or falsification of data, duplicate publication and/or submission, and redundancy.

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Effects of Hydroxychloroquine and Tacrolimus on Discoid Facial Lupus Erythematosus in a Dog

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CASE REPORT
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ABSTRACT

Discoid lupus erythematosus is an autoimmune disease that affects the skin in dogs. 6-year-old male German Shepherd weighing 38 kg was taken to the Small Animal Hospital at the University in Kyiv, Ukraine, with a history of progressive skin lesions. The indications of discoid lupus erythematosus in dogs manifested as red, scaly macules or papules on the skin's surface. These gradually develop into follicular plugging, disc-shaped plaques with adherent scales, and peripheral hyperpigmentation. The oral hydroxychloroquine was used for medication, and the prescribed dosage was 5.0 mg per kilogram of the dog's weight, administered once daily. The hydroxychloroquine was gradually reduced and discontinued within a month. Concurrent with the hydroxychloroquine treatment, the application of tacrolimus cream (Protopic® 0.03%) was initiated. The veterinary physician also advised the dog owner to limit sun exposure to avoid any adverse effects. After a four-week period, there was a decrease in pruritus and erythema, and plaques had flattened although the skin still had some patchy hyperpigmentation. Approximately 3-4 weeks later, the veterinarian determined that the dog had achieved clinical remission as all the skin lesions had become completely flattened. The use of deproteinized calf blood extract gel (Solcoseril® Gel for external 4.15 mg/1 g, Legacy led to the complete disappearance of the initial redness and prevented the appearance of new skin lesions. These results can be considered as a safe and effective alternative to conventional treatment methods.

Keywords: Dog, Discoid lupus erythematosus, Treatment

INTRODUCTION

The first description of the clinical manifestation of hitherto unknown skin disease in a dog that resembled human discoid lupus erythematosus was made in 1979 (Olivry et al., 2018; Marín-García and Llobat, 2022). Later, conflicting data regarding this dermatological pathology were found in the literature, which was reflected in the further improved nosological classification (Kuhn and Landmann, 2014). The first attempts to classify lupus erythematosus were based on the clinical pathology signs, such as systemic bullous type I form, exfoliative, and vesicular form of discoid lupus erythematosus (Salas and Kotschwar, 2014; Lecaros-Cornejo et al., 2015; Fukushima et al., 2021). Pathogenesis of the disease has complex mechanisms of development, which are still thoroughly studied by scientists from different countries of the world (Garelli et al., 2021). The etiology of discoid form lupus is multifactorial and includes environmental exposures, stochastic factors, and genetic susceptibility (Fernandes et al., 2016; Tham et al., 2020). Great progress has been made in understanding the pathogenesis of immune mechanisms (Banovic et al., 2017; Di Cerbo et al., 2021; Amudzi et al., 2022). To understand the mechanisms behind tissue damage and loss of tolerance, investigating lymphocyte signaling, phagocyte function, immune cell apoptosis, and interferon production pathways are crucial areas of study (Zhelavskyi, 2021). A better understanding of the pathogenesis of discoid form lupus has led to renewed interest in targeted therapy, and researchers are now on the cusp of developing targeted immunotherapy (Zhang et al., 2017; Zhelavskyi et al., 2020; Treeful et al., 2022).

Autoimmune reactions lead to the pathogenesis of lupus erythematosus. In a certain way, this creates certain difficulties for doctors in diagnosing and treating animals (Ferrigno et al., 2019; Zhou et al., 2021). The current study presented a novel approach to treating facial discoid lupus erythematosus (FDLE) in a German Shepherd dog, which has not been previously described in the veterinary literature.

MATERIALS AND METHODS

Ethical approval

The dog belonged to private owners, and written informed consent was obtained from them. Clinical studies were conducted in accordance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (No. 3447-IV of

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February 21, 2006) and the requirements of the European Commission for the treatment of vertebrates and protection against thirst, hunger, malnutrition, discomfort, fear, pain, and suffering.

Case presentation

A 6-year-old male German Shepherd weighing 38 kg was brought to the Small Animal Hospital, Kyiv, Ukraine, due to developing skin lesions that gradually worsened. The research was conducted during May-June 2022. These nasal plaques initially appeared as itchy, scaly, erythematous lesions.

Despite conducting blood tests (morphological and biochemical) and numerous screenings for parasites and dermal mycosis, the underlying cause of the dog's clinical symptoms could not be identified. The dog was given various courses of medication, including corticosteroids (prednisolone at a dose of 4 mg/kg orally once a day) and enrofloxacin (4.0 mg/kg orally once daily), for 14 days, leading to some improvement. The clinical signs (hyperemia faces and pruritus) returned immediately upon discontinuation of therapy.

At the beginning of treatment and the dynamics of therapy, a detailed clinical examination of the patient was carried out. Parasitic diseases and dermatomycoses were not verified during the laboratory examination of slides obtained from the affected areas of the body. Treatment (determination of doses and duration of the course) was carried out on the basis of existing protocols (Gutfreund et al., 2013; Rossi et al., 2015). The originality of the research lies in the combination of drugs and the use of means for tissue regeneration.

The dog was given oral hydroxychloroquine (Plaquenil® Sanofi, France) at a dosage of 5.0 mg/kg once a day (Roxane Laboratories), which was gradually reduced and stopped over the course of a month. Concurrently, the dog was also treated with Crème tacrolimus (Protopic® 0.03% Crème, LEO Laboratories Limited, Denmark) and was advised to avoid excessive sun exposure (the cream was applied in a thin layer once a day). After four weeks, the dog's itchiness and redness had disappeared, and the plaques had flattened, although some mottled hyperpigmentation remained. Complete remission was estimated to have occurred in the following 3-4 weeks, as all skin lesions had completely flattened. The application gel deproteinized calf blood extract (Solcoseril® Gel for external 4.15 mg/1 g, Legacy Pharmaceuticals Switzerland, GmbH) stimulated the disappearance of erythema and tissue repair.

Clinical signs

The initial skin lesions are characterized by redness and scaling, which gradually transform into disc-shaped plaques with attached scales, comedones, and darkening around the edges. These plaques combine, causing central scarring and loss of pigmentation (Ferrigno et al., 2019; Zhou et al., 2021). The initial skin manifestations in dogs involve erythema, loss of pigmentation, and scaling, which can advance to erosions with crust formation if the skin surface is disrupted. The lesions typically affect the nose, and dogs may also develop skin lesions on the upper back (Kuhn and Landmann, 2014; Olivry et al., 2018).

The dorsal nasal region was affected by partial baldness and consolidating annular to polycyclic, hyperpigmented plaques with fine silvery scaling (Figure 1 a). The central part of hyperpigmented lesions started to lose the normal skin texture. On the other hand, early skin lesions in canine FDLE typically involve scaling, depigmentation, and erythema, which then progress to erosions and ulcers that result in atrophy and loss of the nasal planum's structure (Figure 1 b). Crusting can occur when there is damage to epithelial integrity. Moreover, skin lesions can have an effect on the nasal planum. Starting from day 5, there was a gradual decrease in hyperemia of the wound and skin around the wound, itching, and pain reaction. From day 10 of treatment, a gradual epithelization of the wounds took place. Complete tissue regeneration was seen on day 30 (Figure 2).

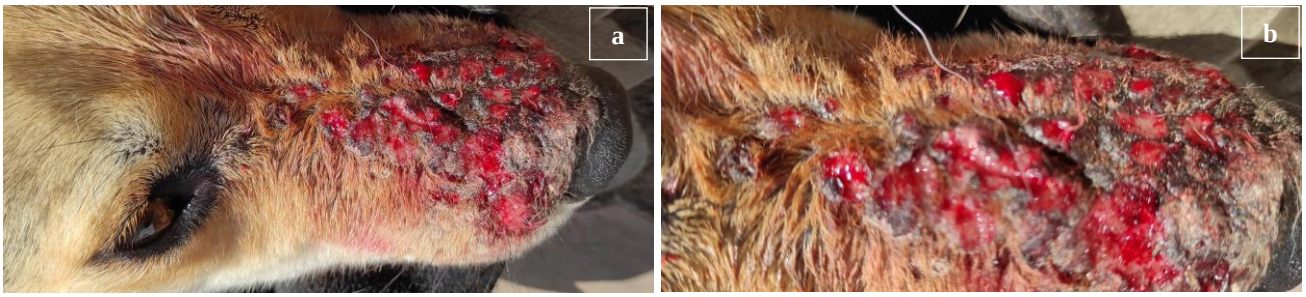

Figure 1. Clinical characteristics of canine facial discoid lupus erythematosus in a German Shepherd dog. **a**: Erythematous, depigmented, ulcerated, and crusted nasal lesions of facial; **b:** Complete loss of cobblestone appearance is visible (magnification 2.5)

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Figure 2. Clinical changes during the treatment of a German Shepherd dog; Starting from day 5, hyperemia of the wound and skin around the wound, itching, and pain reaction gradually decreased. **a:** A gradual epithelization from day 10 of treatment, **b:** Wound healing (day 15), **c:** Tissue repair on day 30

DISCUSSION

Over the past decades, there have been accumulated data on the diagnosis and treatment of canine FDLE in dogs. There are more and more data on immunogenetic factors and the manifestation of autoimmune reactions in this pathology (Chong et al., 2022). Despite this, veterinarians are actively searching for effective methods and means of treatment. For the most part, therapy focuses on the use of drugs that have an immunosuppressive effect. Earlier research has indicated that immunosuppressive doses of corticosteroids are effective in treating patients with discoid lupus erythematosus (Hyun et al., 2021). It has been suggested that the combination of tetracycline and niacinamide, with or without corticosteroids, may be a viable treatment combination (Rossi et al., 2015). In the treatment of discoid lupus erythematosus, cyclosporine (a systemic calcineurin inhibitor) has been administered either as a monotherapy or in combination with glucocorticoids (Banovic et al., 2014). Topical application of tacrolimus has been found to be an effective treatment for autoimmune skin conditions (Gutfreund et al., 2013). The use of tacrolimus in discoid lupus erythematosus of dogs has been reported (Hyun et al., 2021).

The gel form of solcoseryl, a medication derived from protein-free dialysate from calf blood, was applied as a treatment. Solcoseryl increased adhesion, migration, proliferation, and wound healing (Martí-Carvajal et al., 2014).

Open sources provide limited information on the use of solcoseryl in clinical practice. Fragmentary reports are available regarding the treatment of patients with corneal lesions and dermatological problems (Nam and Maen, 2019). The positive dynamics of treatment give prospects for the combined use of drugs (hydroxychloroquine, tacrolimus, and solcoseryl gel) with discoid lupus erythematosus in canis.

In this case, the proposed therapy can be used by clinicians for the therapy of discoid facial lupus erythematosus in dogs. Further research and the development of pharmacology will be an excellent impetus for the development of effective treatments.

CONCLUSION

The treatment of clinical features of facial discoid lupus erythematosus by hydroxychloroquine, tacrolimus, and gel deproteinized calf blood extract ointment in a German Shepherd dog had successful results. No drug-related side effects were seen. These results can be considered in the treatment of facial discoid lupus erythematosus and suggested to be an alternative to previous standard treatments.

DECLARATIONS

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Competing interests
There is no conflict of interest.

Ethical consideration
The authors considered all necessary ethical issues (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy).

Authors' contributions
Mykola Zhelavskyi conceived of the presented idea. Mykola Zhelavskyi and Serhii Kernychnyi verified medical history, contributed to data, carried out the experiment, wrote a manuscript, and prepared the article for submission. Tamara Betlinska participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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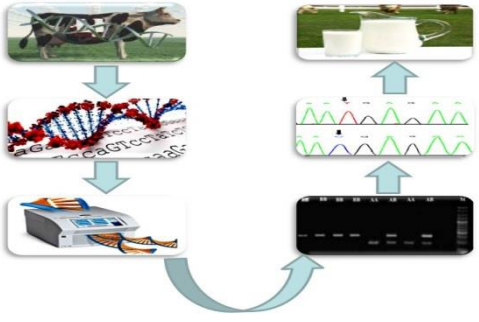

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Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The abstract should be 150 to 300 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and Methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the author(s)'s experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the results but should be put into the discussion section.

Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

Conclusion can be presented jointly if preferred.

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Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph forms or repeated in the text.

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Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the published article. In computational studies where the sequence information is unacceptable for inclusion in databases because of lack of experimental validation, the sequences must be published as an additional file with the article.

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-Examples (at the text- blue highlighted)

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; Chukwura, 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001).

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Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603. Link

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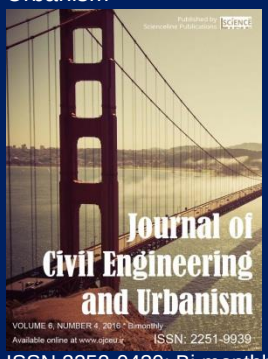

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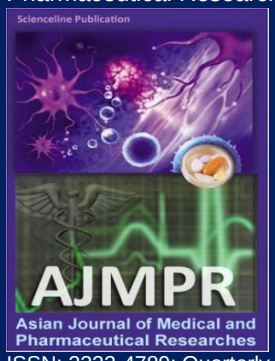

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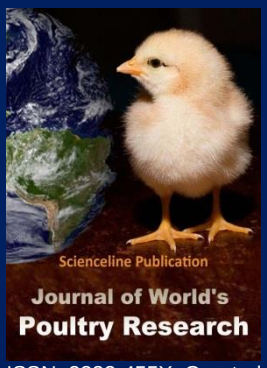

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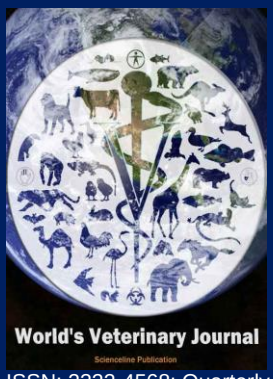

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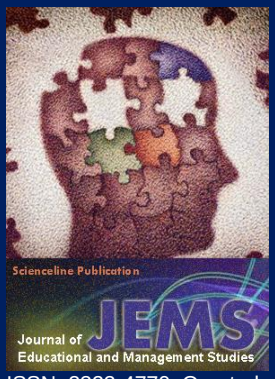

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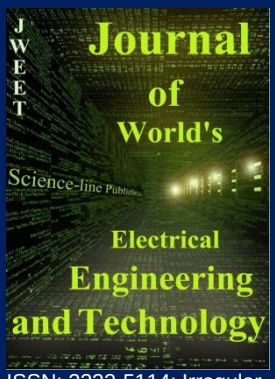

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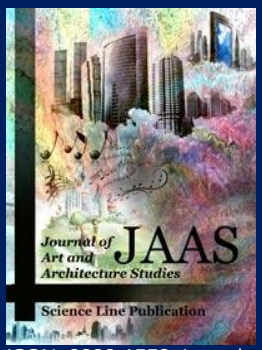

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