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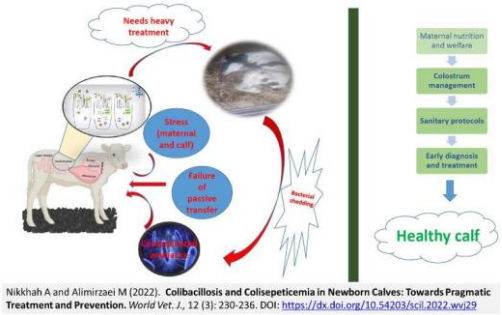
Volume 12 (3); September 25, 2022 [EndNote XML for Agris]

Review

Colibacillosis and Colisepeticemia in Newborn Calves: Towards Pragmatic Treatment and Prevention

World Vet. J. 12(3): 230-236, 2022; pii:S232245682200029-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj29



ABSTRACT: Diarrhea is the most perturbing disease in dairy and beef industries worldwide, leading to significant rates of morbidity and mortality as well as economic losses. The objective of this review article was to delineate the pathophysiology and practical biology of colisepticemia in neonatal calves. Preventive and therapeutic protocols were also presented and discussed from a new integrative perspective. Notably, the situation can be the most deleterious in case diarrhea turns into septicemia. Under such circumstances, the mortality rate may be remarkably high and hard to control. *Escherichia coli* (*E. coli*) is an invasive and opportunistic bacteria causing severe diarrhea (colibacillosis) and colisepticemia in newborn calves. Colisepticemia is commonly prevalent in 2-5 days old calves, and colostral immunity is considered the first defensive line against *E. coli* infection. In addition to colostrum feeding quality and management, other management factors, such as dry cow nutrition and welfare, newborn calf welfare and nutrition, housing system, sanitation protocols, as well as early identification and treatment of sick calves, are important in preventing colisepticemia. In conclusion, understanding the mechanism of action and transmission routes of pathogenic *E. coli* will provide scientific and practical insight to plan preventive and therapeutic protocols decisively and successfully.
Keywords: Diarrhea, Mortality, Newborn calf, Pragmatic Prevention, Septicemia

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Research Paper

Improved Dot-ELISA Assay Using Purified Sheep *Coenurus cerebralis* Antigenic Fractions for the Diagnosis of Zoonotic Coenurosis

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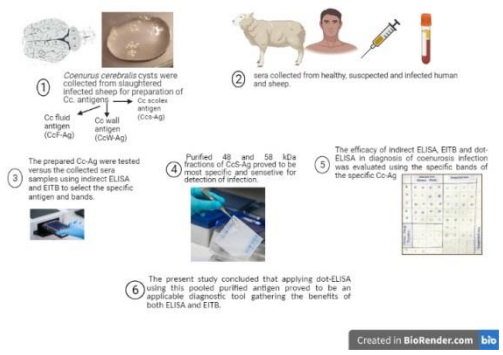
World Vet. J. 12(3): 237-249, 2022; pii:S232245682200030-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj30



ABSTRACT: Clinicians face significant problems in the diagnosis of zoonotic coenurosis. The current study aimed to develop an improved dot-Enzyme-linked-immunosorbent assay (dot-ELISA) for the diagnosis of zoonotic coenurosis using sheep *Coenurus cerebralis* scolices purified antigen (CcS-Ag) and to compare the obtained results with those of indirect ELISA and Enzyme-linked immunoelectrotransfer blot technique (EITB). Sera were collected from humans and sheep infected or suspected of infection with *Coenurus cerebralis*, control cases, and cases infected with other parasites. The CcS-Ag was proved to be the most specific antigen. This antigen was fractionated, and its specific polypeptides against anti-*C. cerebralis* antibodies (ACc-Ab) were identified using EITB. Fractions at the molecular weight (MW) of 48 and 58 kDa were proved as the only specific ones, eluted from the gel and concentrated, then dotted on the NC sheet as pooled antigen before its evaluation in the diagnosis of infection using dot-ELISA. Dot-ELISA demonstrated absolute 100% sensitivity and 100% specificity as recorded by EITB, compared to both fractions on a nitrocellulose (NC) sheet using surgically proved infected human or sheep sera as a gold standard. Diagnosis by ELISA using crude CcS-Ag revealed similar sensitivity but lower specificity (75%). The diagnostic accuracy of dot-ELISA was proved by comparing its results with postmortem data obtained post slaughtering of 20 suspected sheep and patients investigated by computed tomography (CT) and magnetic

resonance imaging (MRI). In conclusion, the selection of specific fractions after EITB to be used in dot-ELISA improved the diagnostic value of the test as a diagnostic tool gathering the benefits of ELISA and EITB.
Keywords: Antigen,*Coenurus cerebralis,* Dot-ELISA, Human, Sheep, Scolices

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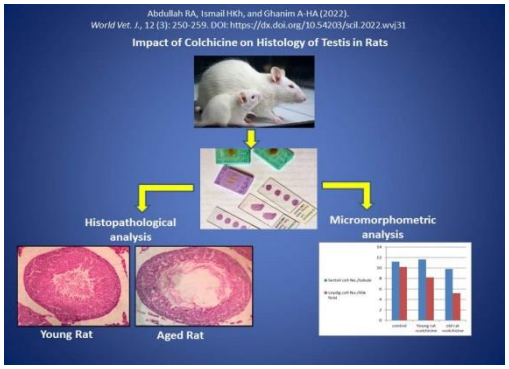
Research Paper

Impact of Colchicine on Histology of Testis in Rats

World Vet. J. 12(3): 250-259, 2022; pii:S232245682200031-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj31



ABSTRACT: Colchicine is a drug widely used for the management of many disorders, such as acute gout and Behçet's disease. It is also prescribed for the treatment of pericarditis, atrial fibrillation coronary artery diseases, and secondary amyloidosis. In case this drug is used at the early stages of coronavirus infection, its anti-inflammatory properties may reduce the severe inflammatory reactions related to a cytokine storm by affecting the inflammasome. The purpose of the present study was to determine the toxicity of Colchicine on testis in rats from different age groups for 10 days. A total of 27 male Wistar rats were divided into three groups. The rats in group I (control group) were administered distilled water by oral gavage. Group II consisted of young rats (5-6 months old) who orally received Colchicine 3 mg/kg body weight. Group III entailed rats of 14-16 months who were orally administered colchicine 3 mg/kg body weight. The testis of the treated groups was dissected and examined for histological changes and morphometrical analysis. The obtained results indicated that high doses of Colchicine (3 mg/kg body weight) could induce tissue damage to the testis, including degeneration and necrosis of both Sertoli and Leydig cells with irregular divisions of germinal epithelium, even when it was used for short periods (10 days). In the elderly treated rats, there were severe tissue damages, including degeneration and necrosis of germinal epithelium with irregular divisions of germ cells, necrosis of Sertoli and Leydig cells with sloughing of germinal epithelium toward the lumen of the tubule. Therefore, there is a need to conduct more studies to investigate the side effect of Colchicine as it is excessively used in the management of coronavirus.
Keywords: Colchicine, Histology, Morphometric trait, Rat, Testis

[Full text-PDF] [Scopus] [Export from ePrint]

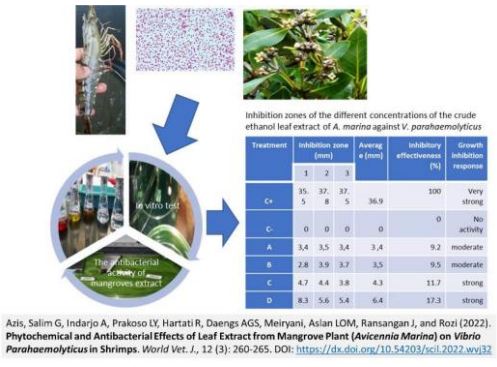
Research Paper

Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on Vibrio Parahaemolyticus in Shrimps

Azis, Salim G, Indarjo A, Prakoso LY, Hartati R, Daengs AGS, Meiryani, Aslan LOM, Ransangan J, and Rozi.

World Vet. J. 12(3): 260-265, 2022; pii:S232245682200032-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj32



ABSTRACT: Recently, there has been a tremendous increase in the studies addressing the application of bioactive compounds from the natural ecosystem, particularly for medical purposes. Hence, the present study investigated the antibacterial properties of the secondary metabolites possibly contained in the leaves of *Avicennia marina* (*A. marina*) for possible prevention of *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a devastating bacterial pathogen in shrimp aquaculture. In the current study, secondary metabolites were extracted from the leaves of mangrove plant using ethanol extraction method. The ethanolic extracts were then subjected to phytochemical and antibacterial activity tests. The results from the phytochemical analysis demonstrated that the ethanolic extract from the mangrove plant contained varying amounts of flavonoids, tannins, saponins, polyphenols, alkaloids, steroids, and triterpenoids. However, the number of flavonoids and alkaloids seemed to be higher than the other metabolites. The antibacterial activity analysis through the agar diffusion method has shown that different concentrations (50 ppm, 100 ppm, 200 ppm, and 300 ppm) of the ethanolic extract of *A. marina* inhibited the *V. parahaemolyticus.* At 300 ppm, the

plant extract exhibited 17.3% antibacterial effectiveness, compared to the antibacterial activity of chloramphenicol. The findings indicated that the secondary metabolites of *A. marina* have the potential that can be developed as an alternative treatment for aquatic animal diseases in the future.
Keywords: Aquaculture, Bioactive compounds, Mangrove ecosystem, Treatment

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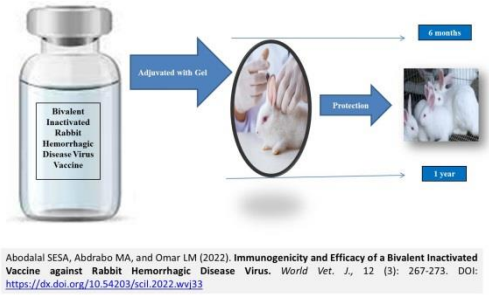
Research Paper

Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus

Abodalal SEA, Abdrabo MA, and Omar LM.

World Vet. J. 12(3): 266-273, 2022; pii:S232245682200033-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj33



Abodalal SESA, Abdrabo MA, and Omar LM (2022). **Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus.** *World Vet. J.*, 12 (3): 267-273. DOI: https://dx.doi.org/10.54203/scil.2022.wvj33

ABSTRACT: Rabbit hemorrhagic disease is a fatal threat to rabbits that causes sustainability problems and substantial economic losses. The aim of the current study was to compare the immuno-enhancing effects of a bivalent inactivated rabbit hemorrhagic disease virus (RHDV) vaccine adjuvanted with Montanide with an inactivated RHDV vaccine with an aluminum hydroxide gel. Montanide incomplete seppic adjuvant 71 VG was prepared as an oil emulsion, and several batches adjuvanted with an aluminum hydroxide gel were prepared. Then, 250 New Zealand rabbits aged 6 weeks were randomly allocated to three groups. Group 1 was subjected to the bivalent inactivated RHDV adjuvanted with an aluminum hydroxide gel, Group 2 received the oil-emulsion vaccine adjuvanted with Montanide, and Group 3 was left unvaccinated as a negative control group. Efficacy was determined using a hemagglutination inhibition test, and resistance was determined using virulent RHDVa and RHDV2. The clinical signs included sudden death, nervous manifestations, aimless running, lateral recumbence, and crying before death. The mortality rates were recorded at 3 weeks, 3 months, 6 months, and 12 months after vaccination. In addition, blood samples were collected on the first day as well as 1, 2, 3, 4, 6 weeks post-vaccination (WPV), and 2, 3, 4 month post-vaccination (MPV) until 12 MPV. Serological analysis indicated that the bivalent inactivated RHDV oil-emulsion vaccine was more effective than the bivalent inactivated RHDV aluminum hydroxide gel vaccine, resulting in improved immune responses and longer-lasting protective immunological responses in vaccinated rabbits. The bivalent inactivated RHDV oil-emulsion vaccine was also sterile and safe and helped the protection of the rabbits against RHDVa and RHDV2, hence reducing the time and effort required during the vaccination process and reducing the levels of discomfort for the rabbits.
Keywords: Immunity, Inactivated vaccine, Oil emulsion, Rabbit hemorrhagic disease virus

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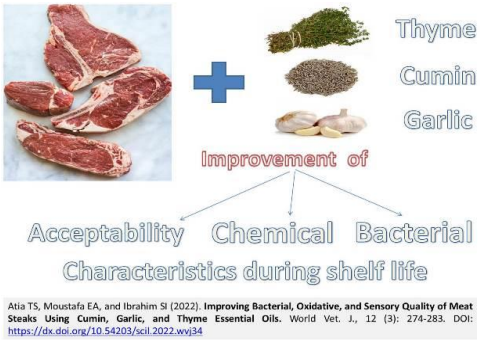
Research Paper

Improving Bacterial, Oxidative, and Sensory Quality of Meat Steaks Using Cumin, Garlic, and Thyme Essential Oils

Atia TS, Moustafa EA, and Ibrahim SI.

World Vet. J. 12(3): 274-283, 2022; pii:S232245682200034-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj34



Atia TS, Moustafa EA, and Ibrahim SI (2022). **Improving Bacterial, Oxidative, and Sensory Quality of Meat Steaks Using Cumin, Garlic, and Thyme Essential Oils.** World Vet. J., 12 (3): 274-283. DOI: https://dx.doi.org/10.54203/scil.2022.wvj34

ABSTRACT: The meat industry increasingly considers meat shelf life as a critical problem. Some essential oils contain antibacterial and antioxidant characteristics that help to keep meat safe. Therefore, the purpose of this study was to evaluate the preservation benefits, including antibacterial and antioxidant properties, of cumin, garlic, and thyme essential oils at 1% on chilled beef meat steaks, as well as their effects on pH, total volatile basic nitrogen (TVBN), thiobarbituric acid (TBA), and related sensory aspects (color, odor, appearance, consistency, and overall acceptability). The results of the current study showed that pretreating beef meat steaks with EOs of cumin, garlic, and thyme at a concentration of 1% effectively reduced levels of APC, coliform count, *staph aureus* count, TVBN, and TBA while extending shelf life to 12, 15, and 18 days when stored at 4°C. In terms of antibacterial and antioxidant properties, shelf life, and sensory quality on beef meat steaks, the thyme essential oil group outperformed cumin and garlic essential oils. The current study introduced an effective natural preservative alternative that could replace undesirable synthetic substances in the future while also lowering antibiotic resistance.

Keywords: Coliforms, Cumin, Garlic, Preservation, Shelf life, Thyme

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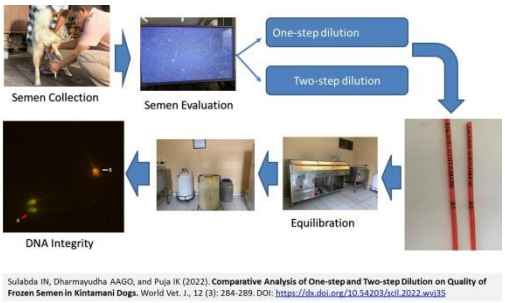
Research Paper

Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs

Sulabda IN, Dharmayudha AAGO, and Puja IK.

World Vet. J. 12(3): 284-289, 2022; pii:S232245682200035-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj35



Sulabda IN, Dharmayudha AAGO, and Puja IK (2022). **Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs.** World Vet. J., 12 (3): 284-289. DOI: https://dx.doi.org/10.54203/scil.2022.wvj35

ABSTRACT: Preservation of sperm by freezing allows breeding dogs that are separated over long distances. To increase the fertility of frozen and then thawed spermatozoa, they must be able to survive the process. The current study aimed to evaluate the sperm motility and DNA integrity of Kintamani dogs extended in extenders with one-step and two-step dilution techniques. Ejaculates collected from four dogs were used in the current study. The semen was divided into two equal parts and diluted with extenders using two different dilution techniques, namely One-step dilution in Tris egg yolk containing 7% glycerol, and a two-step dilution technique diluted in an initial 2:1 with an extender, containing 20% egg yolk without glycerol. The same volume of the second extender was added, including 14% glycerol. The sample was loaded into 0.25 ml straws, cooled to 4°C for 4 hours, equilibrated, and then plunged into the liquid nitrogen. The sperm motility was evaluated using Computer-Assisted Sperm Analysis (CASA), and DNA integrity was assessed using Acridine Orange (AO) stained. Results showed that the sperm motility of Kintamani dogs in extenders using two-step dilution was significantly higher compared to the one-step dilution technique. In addition, the obtained results indicated that two types of dilution steps in Kintamani dog semen were not detrimental to the sperm DNA integrity during the freezing process. In conclusion, extenders with two types of dilution techniques could maintain sperm motility above 30%, and no difference between one and two steps dilution was detected.
Keywords: Dilution techniques, DNA integrity, Egg yolk, Kintamani dog, Motility

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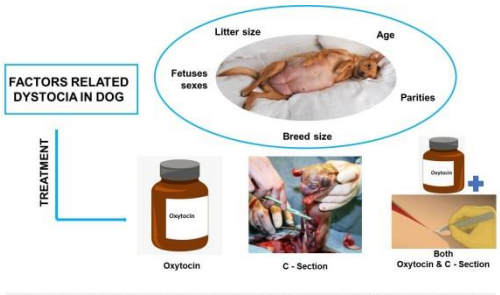
Research Paper

Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam

Long STh, Hien NThTh, Hang PhTh, Hoai NTh, and Bach PhX.

World Vet. J. 12(3): 290-295, 2022; pii:S232245682200036-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj36



Long STh, Hien NThTh, Hang PhTh, Hoai NTh, and Bach PhX (2022). **Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam.** *World Vet. J.*, 12 (3): 290-295. DOI: https://dx.doi.org/10.54203/scil.2022.wvj36

ABSTRACT: Dystocia is a common disorder that can cause harmful health risks to bitch and puppies. The aim of the current study was to evaluate some risk factors related to canine dystocia and the application of treatment methods to 612 diagnosed cases in Gaia Pets Clinic and Resort, Hanoi, Vietnam, from December 2013 to May 2020. The investigated factors comprised age, parity and breed size, and litter size, as well as fetal sex in relation to the proportion of dystocia in female canines. Dystocia was frequently observed in female dogs aged 1-3 years, with rates of 76.1%. The highest proportion of dystocia was found in the first litter group (80.21%). The incidence of dystocia increased as the weight of the dog decreased, and it was prevalent in the small breed (61.93%). Dystocia risk decreased as the litter size increased. The interventions used in this study were medical treatment with the hormone oxytocin (1.8%), surgical management with cesarean section (86.11%), and a combination of oxytocin and cesarean section (12.09%), with the success rates of each treatment method as 100%, 98.86%, and 100%, respectively. Some risk factors, such as age, parity, breed size, and litter size identified in the present research, could be used as prognostic indicators in the veterinary practice to optimize the survival rate of female dogs and puppies.
Keywords: Age, Breed, Dystocia, Fetus sex, Litter size, Parities

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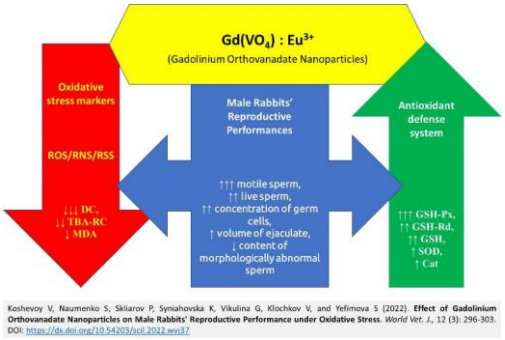
Research Paper

Effect of Gadolinium Orthovanadate Nanoparticles on Male Rabbits' Reproductive Performance under Oxidative Stress

Koshevoy V, Naumenko S, Skliarov P, Syniahovska K, Vikulina G, Klochkov V, and Yefimova S.

World Vet. J. 12(3): 296-303, 2022; pii:S232245682200037-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj37



ABSTRACT: Oxidative stress as a leading factor of male infertility requires correction with modern pharmacological agents, particularly redox-active nanoparticles, to improve sperm quality and hormonal balance. The current experimental study aimed to investigate the effect of orthovanadate nanoparticles of rare earth elements, particularly Gadolinium, with pronounced redox properties on the reproductive function of male rabbits under oxidative stress. A total of 36 mature male Hyla rabbits were divided into three groups of intact control (n = 12) and two experimental groups, including rabbits ubder oxidative stress (n = 12), induced by the introduction of tert-Butyl hydroperoxide, and those under oxidative stress plus hydrosol of gadolinium orthovanadate nanoparticles (NPs, n = 12) intake for 14 days. There were four rabbits per three replicates in each group. Animals of all groups were kept on the same diet and had free access to water. The use of NPs led to an improvement in sperm quality indicators. There was an improvement in motility and ejaculate volume indicators (by 14.6% and 39.2%, respectively), a reduction of the content of morphologically abnormal sperm by 26.7%; normalization of sex hormones, an increase in the level of total testosterone (by 113%) with a decrease in 17-β-estradiol (by 16.5%). This sex hormones improvement led to an increase in the androgen saturation of the rabbit's body (free androgen index at the end of the experiment was 36.5%). The obtained changes were accompanied by a decrease in the oxidative load, as evidenced by a reduced content of diene conjugates and thio-barbituric acid-reactive compounds in the blood serum of rabbits by 30.4% and 26.8%, compared to the control. At the same time, there was an increase in the antioxidant potential, especially its glutathione link – the activity of glutathione peroxidase and glutathione reductase (by 42.5% and 34.2%, respectively), and the content of reduced glutathione increased by 62.3%, compared to the indicators before the introduction of NPs. The results of the study confirmed the effectiveness of using gadolinium orthovanadate NPs to correct the reproductive function of males under oxidative stress.
Keywords: Gadolinium orthovanadate, Male rabbits, Nanoparticles, Oxidative stress, Reproductive performances

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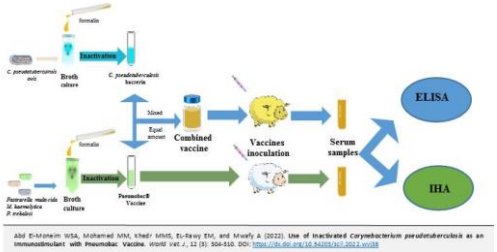
Research Paper

Use of Inactivated Corynebacterium pseudotuberculosis as an Immunostimulant with Pneumobac Vaccine

Abd El-Moneim WSA, Mohamed MM, Khedr MMS, EL-Rawy EM, and Mwafy A.

World Vet. J. 12(3): 304-310, 2022; pii:S232245682200038-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj38



ABSTRACT: Sheep breeders in Egypt suffer from pneumonic pasteurellosis caused by *Pasteurella trehalosi, Pasteurella multocida*, and *Mannheimia haemolytica*. The disease is responsible for significant economic losses in the sheep industry according to the high mortality rate and reduced carcass values. Pneumobac® is the primary vaccine in Egypt used to control pasteurellosis in sheep. Therefore, the aim of the present study was to estimate the nonspecific immune stimulating impact of *Corynebacterium pseudotuberculosis ovis* against *Pasteurella* in sheep vaccinated with Pneumobac®. Nine sheep were classified into three groups, each with three animals. The sheep in the first and second groups were inoculated with the inactivated culture of Pneumobac® and a combined inactivated culture of Pneumobac® with *Corynebacterium pseudotuberculosis ovis* bacterin, respectively. The third group was nonvaccinated and kept in control. Indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) were used to measure the humoral immune response to the produced vaccines. The results of the present study confirmed that the antibodies titer against *Pasteurella multocida* type A, D, and B6, *Pasteurella trehalosi* type T, and *Mannheimia haemolytica* type A significantly increased in sheep vaccinated with a combined vaccine (Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin), compared to those vaccinated with Pneumobac® alone. It was concluded that the

addition of *Corynebacterium pseudotuberculosis ovis* bacterin to inactivated Pneumobac® vaccine could increase the immune response against pneumonic pasteurellosis.
Keywords: *Corynebacterium pseudotuberculosis*, *Pasteurella multocida*, Pasteurellosis, Pneumobac®

[Full text-PDF]

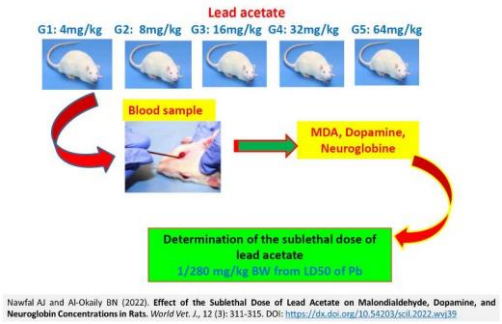
Research Paper

Effect of the Sublethal Dose of Lead Acetate on Malondialdehyde, Dopamine, and Neuroglobin Concentrations in Rats

Nawfal AJ and Al-Okaily BN.

World Vet. J. 12(3): 311-315, 2022; pii:S232245682200039-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj39



ABSTRACT: Lead can have detrimental behavioral, biochemical, and physiological effects on the body. The current experiment was designed to estimate the sublethal dose of lead acetate that induce oxidative stress on the central nervous system (CNS) in adult using the probit analysis. Moreover, the current study examined the dose-response curve by successive doses of lead acetate on some parameters related to oxidative stress for 28 days. A total of 36 adult male rats were randomly selected and divided equally into six experimental groups and treated for 28 days. Rats in the control group received distilled sterile water, and those in G1, G2, G3, G4, and G5 were gavaged with 4, 8, 16, 32, and 64 mg/kg of lead acetate, respectively. The result indicated a positive correlation between the successive doses of lead acetate. Malondialdehyde concentration decreased dopamine and neuroglobin by increasing the dose of lead acetate in experimental groups (G3, G4, and G5), compared to the control group. In conclusion, exposure to the sublethal dose of 16 mg/kg of lead acetate significantly alters the levels of the neurotransmitters and increases the production of oxidative stress in the CNS tissue.
Keywords: Central nervous system, Dopamine and Neuroglobin, Lead acetate, Malondialdehyde, Rat

[Full text-PDF]

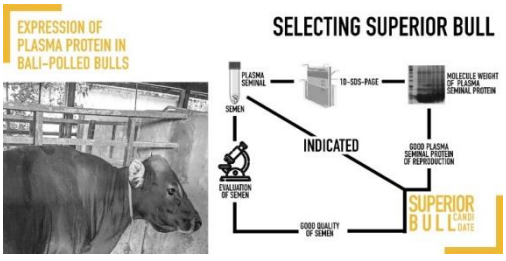
Research Paper

The Expression of Plasma Protein in Bali-polled Bulls Using 1D-SDS-PAGE

Diansyah AM, Yusuf M, Toleng AL, Dagong MIA, and Maulana T.

World Vet. J. 12(3): 316-322, 2022; pii:S232245682200040-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj40



ABSTRACT: The fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. Factors, such as the interstice factor of the sperm and the plasma component of semen, affect the fertility rate of bulls. The fertility rate can also be determined by identifying the protein content of semen plasma. Therefore, the current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bulls. The study was conducted at the Laboratory of Semen Processing, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia, the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia from November 2021 to January 2022. The samples came from 5 Bali-polled and 5 Bali-horned bulls. Semen collection was conducted twice a week using an artificial vagina. The concentration of seminal plasma protein was determined by the Bradford method of 1D-SDS-PAGE. The study results showed that fresh semen of Bali-polled and Bali-horned bulls was considered a normal category. Seminal plasma proteins of Bali-polled and Bali-horned bulls were classified using 8 bands to categorize molecular weight; 150 kD (IGF-1), 110 kD (A-kinase anchoring protein 3), 93 kD (A-kinase anchoring protein 4), 54-87 kD (Arylsulfatase-a), 44-62 kD (N-Acetyl-ß-Guicosaminidase), 44kD (Phosphoglycerate kinase), 15-30 kD (BSP A1/A2, BSP-A3 and BSP-30 [BSP1, BSP3, and BSP5]) and 12-14 kD (Acidic seminal fluid proteins). The findings indicated that both Bali-polled and Bali-horned bulls could have a high reproductive rate. In conclusion, protein analysis based on molecular weight using 1D-SDS-PAGE can be used as a biomarker for semen quality in Bali-polled bulls. Therefore, evaluating the semen quality with a molecular basis as an additional indicator of superior bull in the selection process is an alternative method.

Keywords: Bali-polled bull, Seminal protein plasma, Sperm, 1D-SDS-PAGE

[Full text-PDF]

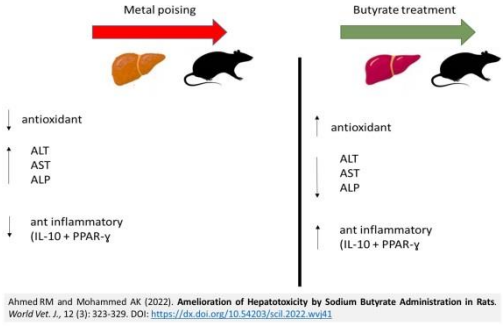
Research Paper

Amelioration of Hepatotoxicity by Sodium Butyrate Administration in Rats

Ahmed RM and Mohammed AK.

World Vet. J. 12(3): 323-329, 2022; pii:S232245682200041-12

DOI:https://dx.doi.org/10.54203/scil.2022.wvj41



ABSTRACT: Lead poisoning is a serious environmental issue with life-threatening consequences. Lead poisoning increases the risk of cancers, gastrointestinal disorders, hepatotoxicity, central nervous system diseases, nephropathy, and cardiovascular diseases in animals and humans. The current study aimed to investigate the effect of sodium butyrate, as an antioxidant, on protecting female adult rats from the harmful effects of lead acetate. A total of 40 adult female albino rats were divided randomly into four equal groups. The first group dealt as the control. The second group received lead acetate at a dose of 200 mg/kg daily orally. The third group received lead acetate at a dose of 50 mg/kg daily orally, and the fourth group received both sodium butyrate and lead acetate orally/day for 35 days. The result indicated that sodium butyrate reduced the concentration of liver enzymes (ALT, AST, and ALP) which were elevated by lead acetate poising. Moreover, sodium butyrate ameliorates the redux status by decreasing malondialdehyde and increasing total antioxidant capacity. Additionally, sodium butyrate-treated rats showed significant alterations in the expression of peroxisome proliferator-activated receptor gamma and interleukin -10 genes. In conclusion, this study reveals an unrecognized role for peroxisome proliferator-activated receptor gamma and Interleukin-10 signaling after sodium butyrate treatment in regulating the immunopathology that occurs during lead acetate poising.
Keywords: Interleukin-10, Lead acetate toxicity, Sodium butyrate, PPAR-gamma, Rat

[Full text-PDF]

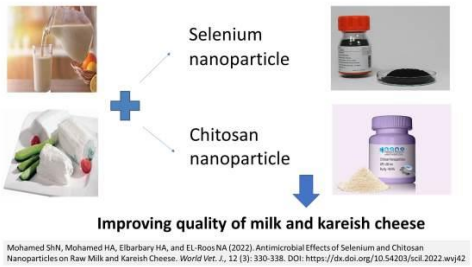
Research Paper

Antimicrobial Effects of Selenium and Chitosan Nanoparticles on Raw Milk and Kareish Cheese

Mohamed ShN, Mohamed HA, Elbarbary HA, and EL-Roos NA.

World Vet. J. 12(3): 330-338, 2022; pii:S232245682200042-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj42



ABSTRACT: The contamination of milk and its dairy products with different microorganisms could cause public health hazards. Antibacterial nanoparticles (NPs) are a novel way to ensure that milk and milk products are safe. The present study investigated the effect of chitosan NPs (CS-NPs) and selenium NPs (Se-NPs) on some microorganisms, which consequently affect raw milk and Kareish cheese. Small-sized nanomaterials of Se-NPs and CS-NPs at the size of approximately 20 nm were used in this study. The samples were 700 ml raw milk and 700g Kareish cheese manufactured from 3000 mg milk. The concentrations of used nanoparticles were 0.5%, 1%, and 1.5% for Se-NPs and 2.5%, 5%, and 10% for CS-NPs. They were used to improve the microbial properties of milk and Kareish cheese samples during storage at the refrigerated temperature of 4°C. The aerobic plate count, Enterobacteriaceae count, Staphylococcus count, and mold count were significantly reduced in milk and Kareish cheese samples treated with CS-NPs and Se-NPs. The study has confirmed that CS-NPs and Se-NPs indicated high antimicrobial activity against the studied microorganisms at all concentrations although CS-NPs were more effective than Se-NPs. It can be concluded that these NPs can be used as preservatives in milk and milk products, such as Kareish cheese. In addition, increasing the concentrations of these NPs by 10% for CS-NPS and 1.5% for Se-NPS boosted their effects.
Keywords: Chitosan, *Enterobacteriaceae,* Kareish cheese, Nanoparticle, Selenium, *Staphylococcus aureus*

[Full text-PDF]

Research Paper

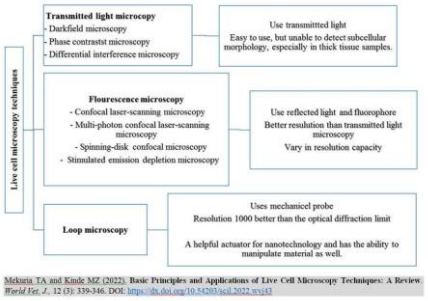
Basic Principles and Applications of Live Cell Microscopy Techniques: A Review

Mekuria TA and Kinde MZ.

World Vet. J. 12(3): 339-346, 2022; pii:S232245682200043-12

DOI: https://dx.doi.org/10.54203/scil.2022.wvj43

ABSTRACT: Live cell imaging has provided great benefits in studying multiple processes and molecular interactions within and/or between cells. This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications. A wide range of microscopic techniques, from conventional transmitted light to an array of fluorescence microscopy techniques, including advanced super-resolution techniques, can be applied for live-cell imaging. Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. On the other hand, fluorescence microscopy uses reflected light to capture images of cells or molecules that have been fluorescently dyed. Techniques for transmitted light microscopy are simple to use but have poor resolution. Although the resolution of fluorescent microscopy techniques is only approximately 200-300 nm, this is nevertheless an improvement over conventional transmitted methods. Conventional light microscopy's resolution was improved by the introduction of the super-resolution microscopy technology family. These methods "break" the diffraction limit, enabling fluorescence imaging with resolutions up to ten times higher than those possible with traditional methods. Each live cell imaging method has advantages and drawbacks. The primary deciding criteria for choosing the type of microscope are the study's objectives, previous experience, the researcher's interests, and financial viability. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount in life science studies.



Keywords: Application, Fluorescence, Imaging, Laser-Scanning, Live cell, Microscopy

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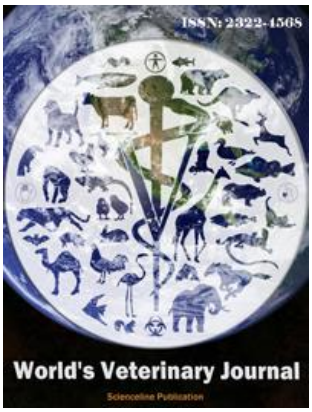

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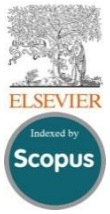

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Colibacillosis and Colisepeticemia in Newborn Calves: Towards Pragmatic Treatment and Prevention

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REVIEW ARTICLE
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ABSTRACT

Diarrhea is the most perturbing disease in dairy and beef industries worldwide, leading to significant rates of morbidity and mortality as well as economic losses. The objective of this review article was to delineate the pathophysiology and practical biology of colisepticemia in neonatal calves. Preventive and therapeutic protocols were also presented and discussed from a new integrative perspective. Notably, the situation can be the most deleterious in case diarrhea turns into septicemia. Under such circumstances, the mortality rate may be remarkably high and hard to control. *Escherichia coli* (*E. coli*) is an invasive and opportunistic bacteria causing severe diarrhea (colibacillosis) and colisepticemia in newborn calves. Colisepticemia is commonly prevalent in 2-5 days old calves, and colostral immunity is considered the first defensive line against *E. coli* infection. In addition to colostrum feeding quality and management, other management factors, such as dry cow nutrition and welfare, newborn calf welfare and nutrition, housing system, sanitation protocols, as well as early identification and treatment of sick calves, are important in preventing colisepticemia. In conclusion, understanding the mechanism of action and transmission routes of pathogenic *E. coli* will provide scientific and practical insight to plan preventive and therapeutic protocols decisively and successfully.

Keywords: Diarrhea, Mortality, Newborn calf, Pragmatic Prevention, Septicemia

INTRODUCTION

Diarrhea and septicemia have long been recognized as major causes of economic damage to neonatal management due to significant rates of morbidity and mortality. Nowadays, despite many advances in veterinary sciences, calf loss in the early stages of life is still a major concern. Unfortunately, often farmers and calf-raising staff are not aware of the interactions in disease agent behavior, host response, and environment. Thus, they are not able to prevent disease outbreaks or treat patients appropriately. In the case of calf diarrhea and colisepticemia, describing the disease's pathophysiology to farm personnel is critical because workers should be educated on how to manage sepsis cases (Nikkhah and Alimirzaei, 2022a).

Generally, diarrhea can be classified into two forms of infectious and non-infectious or nutritional diarrhea (Barteis et al., 2010). Infectious diarrhea, a major cause of neonatal calf mortality, is caused by a wide range of microorganisms (bacteria, viruses, and protozoa) with different severities. Severe cases can lead to septicemia, coma, and death. Sudden death can be a direct consequence of septicemia regardless of whether diarrhea occurs or not (Bashahum and Amina, 2017). Septicemia, caused by pathogenic *E. coli*, is the main fatal condition in newborn calves (Bashahum and Amina, 2017). The pathogenic *E. coli* is a gram-negative bacteria invading calves. It damages small intestine cells at very young ages (2-5 d of age) when the immune system is weak. Thus, *E. coli* have a chance to overgrow and produce toxins (Constable, 2004). It has been reported that the *E. coli* number could be increased 5-10000 fold in the duodenum, jejunum, and ileum of calves with diarrhea (Constable, 2004). When bacterial toxins reach the bloodstream, they cause severe systemic inflammation and trigger subsequent cascade reactions that lead to organ failure and finally death.

Host immunity and environmental contamination are two critical factors determining the calf's susceptibility to infectious diseases in the first week of life (Nikkhah and Alimirzaei, 2022b). Unhygienic calf ambiance accompanied by a weakened immune system due to poor colostrum feeding management could result in severe diarrhea or even septicemia during the first week of life (Nikkhah and Alimirzaei, 2022b). Since moderate cases of diarrhea can be treated successfully, decreasing the number of sepsis calves is considered the first step in controlling diarrhea-related calf mortalities. In addition, providing a comfortable place for both the dam in the late-pregnancy period and neonatal calf would be an appropriate management practice to reduce calf susceptibility to infectious diseases. As the diarrhea-inducing mechanisms and the importance of prevention protocols have been less understood in dairy farms, addressing

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the mechanisms of action for colisepticemia incidence will provide a practical framework to control and treat neonatal calf diarrhea pragmatically.

E. coli PATHOGENICITY

Although *E. coli* is thought to be a fully harmful bacteria, not all of its serotypes are pathogenic (Kaper et al., 2004). *E. coli* is a natural inhabitant of the calf's gastrointestinal tract and can be an invasive agent if the host and environmental conditions favor its replication (Croxen and Finlay, 2010). Unfortunately, many farmers or even farm experts are not aware of the mechanisms that are involved in newborns' diarrhea or septicemia development, hence making it difficult to treat or prevent the problem. Colibacillosis is a common consequence of pathogenic *E. coli*, frequently seen in newborn calves (Croxen and Finlay, 2010). Pathogenic *E. coli* is responsible for extra-intestine infections such as coliseptisemia as well as urinary tract and nervous system issues (Croxen and Finlay, 2010). In this case, pathogenic *E. coli* can be divided into two main pathotypes including diarrhoeagenic *E. coli* and extra-intestinal *E. coli* (Kaper et al., 2004). According to virulence attributes, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E.coli* (EIEC), Enteroaggregative *E.coli* (EAEC), and Diffusely Adherent *E. coli* (DAEC) are six well-described serotypes for diarrhoeagenic *E. coli* (Kaper et al., 2004; Croxen and Finlay, 2010).

Extra-intestinal *E. coli* includes two common serotypes, uropathogenic *E. coli* and (UPEC) and neonatal meningitis *E. coli* (NMEC, Kaper et al., 2004). The major *E. coli* serotypes that infect newborn calves and cause severe diarrhea and colisepticemia are EPEC, ETEC, and EHEC (Janke et al., 1980; Kaper et al., 2004; Bashahum and Amina, 2017). As noted, the pathogenicity of such strains is mediated through plasmid-encoded virulence factors, enabling them to attach to intestinal cells (Janke et al., 1980; Croxen and Finlay, 2010). For instance, ETEC expresses fimbriae or pili to adhere intestinal surface without disrupting mucosal epithelium. It has been demonstrated that ETEC expresses several types of pili, such as K88, K99, 987P, and F41, to attach to intestinal cells (Janke et al., 1980; Bashahum and Amina, 2017). The *E. coli* K99 is believed to be the most common strain inducing colibacillosis in newborn calves.

Following attachment, enterotoxins produced by the bacteria trigger epithelial cells to secrete fluids into the lumen and result in diarrhea (Acres, 1985; Zhang et al., 2022). Heat-labile (LT), heat-stable (STa and STb) toxins are produced by ETEC following adherence and subsequent colonization. Altering ion exchange across the intestinal cells is likely to be the mechanism of ETEC to induce diarrhea. It has been reported that the LT toxin stimulates the cAMP pathway leading to secrete Cl^- into the lumen of small intestine while inhibiting Na^+ absorption (Fleckenstein et al., 2019). On the other hand, ST toxin triggers cGMP accumulation inside epithelial cells. Similarly, cGMP limits Ns/H ion exchanger channel (Fleckenstein et al., 2019). Accordingly, accumulation of salt can lead water into the intestine lumen resulting in diarrhea. The mechanism of ETEC action is schematically summarized in Figure 1.

Enteropathogenic *E. coli* is another cause of diarrhea in newborn calves that results in death. The development of 'attaching and effacing' lesions (A/E) (a histopathologic lesion) on the intestinal cell surface is the most important characteristic of EPEC (Knutton et al., 1993). The attaching bacteria efface microvilli and disturb its action, causing distinct pedestals beneath the site of adherence (Croxen and Finlay, 2010). In addition, EPEC uses pili named bundle-forming pili to attach epithelial cells. Concurrently, bundle-forming pili are used to interact with other EPEC to create localized adherence (Hyland et al., 2008). Enteropathogenic *E. coli* expresses adhesive proteins (tir and intimin) to attach host cells intimately. Pedestal structure and its related factors are schematically described in Figure 2. Moreover, EPEC possesses some multifunctional effectors that translocate into the host cells and subvert normal cell processes, such as mitochondria structure and function, failure of phagocytosis, and tight junctions dysfunction (Ma et al., 2006; Quitlard et al., 2006; Guttman et al., 2006). Attaching and effacing are the main mechanisms inducing diarrhea in newborn calves. Notably, maldigestion and malabsorption of nutrients resulting from effacing of intestine microvilli may exacerbate the health status of diarrheic calves.

Shiga toxin-producing *E. coli* (STPE) is another important bacteria in the etiology of calf diarrhea. In comparison with ETEC and EPEC, STPE pathogenesis has not been comprehensively investigated in calves. Nonetheless, it has been demonstrated that *E. col* 026 and 0111 are responsible for diarrhea in calves (Lee et al., 2008). It belongs to a family of shiga toxin-producing bacteria (Bashahum and Amina, 2017), and similar to other types of *E. coli*, EHEC can infect calves of less than 2 weeks of age. In humans, diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) are frequently seen, whereas diarrhea and dysentery are common in calves (Sandhu and Gyles, 2002). Virulence attributes of STEE are related to toxin production and intestinal colonization (Sandhu and Gyles, 2002). Investigating STEC adherence properties has revealed A/E lesions in the intestinal epithelial cells of calves (Sandhu et al., 1999; Bashahum and Amina, 2017). It is important to note that other diarrhea causes may induce similar results, so it should not be confused with *E. coli*-induced diarrhea. A differential diagnosis is needed to determine the exact origin of the infection. Nonetheless, diarrhea and septicemia during the first week of life are mostly associated with *E. coli* in dairy and beef breeds (Bashahum and Amina, 2017).

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Practically, loss of water and electrolyte imbalance induce some hematological and metabolic alterations, resulting in metabolic acidosis, coma, and death (Dratwa-Chalupnik et al., 2012). Hyponatremia, hypochloremia, and hyperkalemia are common consequences of intestinal damage in neonatal calves (Dratwa-Chalupnik et al., 2012). Furthermore, malabsorption of nutrients such as glucose implicates energy deficits and exacerbates calf general health (Bashahum and Amina, 2017). Correcting such conditions by replacing nutrients and electrolytes lost during diarrhea is vital for establishing normal body homeostasis.

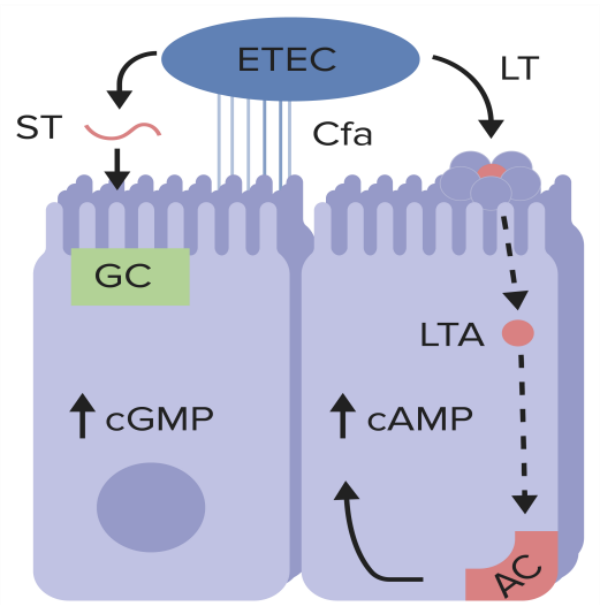

Figure 1. Attachment of Enterotoxigenic *E. coli* (ETEC) and water loss. CFa: Colonization factor antigen; cAMP: Cyclic adenosine monophosphate; GC: Guanylyl cyclase; AC: Adenylyl cyclase

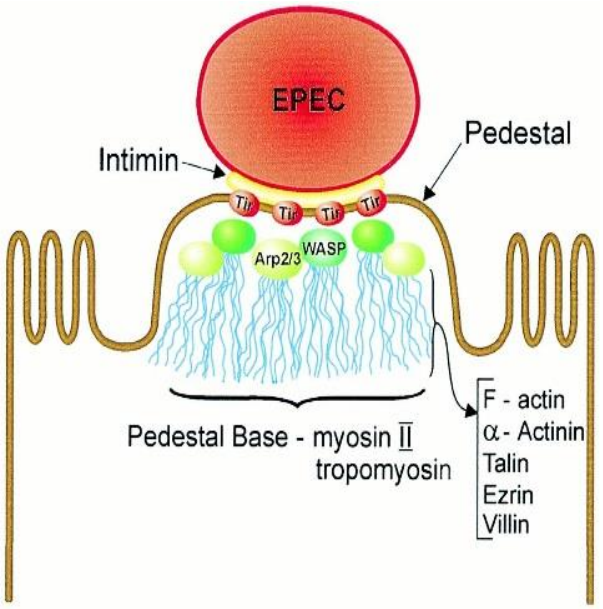

Figure 2. Pedestral structure in Enteropathogenic *E. coli* (EPEC) infection (Vallace and Finlay, 2000). Tir: bacteria's own receptor; Arp2/3: and WASP: Protein complexes recruited towards pedestal tip.

COLISEPTICEMIA AND RELATED CONSEQUENCES

Septicemia is an acute invasion of systemic circulation by bacteria and occurs when bacteria enter the bloodstream and circulate throughout the body after replication (Calfcare.ca). In new definitions, sepsis is considered a life-threatening dysfunction of body organs resulting from dysregulated response to infectious agents (Wasyluk and Zwolak, 2021). In such definitions, nutrient metabolism is emphasized in addition to the inflammatory response addressed in the traditional definition. Bacteria are considered the main cause of septicemia in neonatal calves. The *E. coli-induced* septicemia and colisepticemia, are responsible for high rates of calf mortality in dairy farms worldwide (Vallance and Finlay, 2000; Bashahum and Amina, 2017). All *E. coli* strains described above, ETEC, EPEC, and STEC, have the potential to cause colisepticemia. Signs can vary from severe diarrhea to sudden death without any clinical symptoms (Bshahum and Amina, 2017). With respect to the aggressive nature of colisepticemia, observing some clinical symptoms such as recumbency, suckling inability, and sudden and severe diarrhea in 2-5 d old calves can lead farmers to suspect colisepticemia (Figure 3). Immediate treatment might be effective in saving sepsis calves. If the sepsis time period is extended and invading of bacteria is not controlled, joint arthritis and meningitis are expected due to infectious agent localization (Fecteau et al., 2009).

Figure 3. A two day old recumbent calf (captured by the authors)

A sepsis calf undergoes many physiological abnormalities, resulting in compromised body homeostasis, uncontrolled inflammatory response, organ failure, shock, and death (Vallance and Finlay, 2000). Inflammation is a direct consequence of bacterial invasion to the host tissues. If the local inflammation spreads and the infectious agent has an opportunity to enter into circulation, systemic inflammation may occur (Jaffer et al., 2010). Systemic inflammatory response syndrome (SIRS) and organ dysfunction occur following systemic inflammation, leading to shock and death

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(Jaffer et al., 2010). In other words, SIRS reflects uncontrolled inflammatory responses observed in sepsis cases. The inflammatory response is controlled by pro- and anti-inflammatory cytokines which work together to determine the course of the disease (Bone, 1996). It has been reported that the number of these cytokines is associated with the occurrence of SIRS (Jaffer et al., 2010). Lipopolysaccharide (LPS) of gram-negative bacteria is one of the most powerful stimuli to produce pro-inflammatory cytokines and develop systemic inflammation. Interleukin 1 and TNF-α are the main pro-inflammatory cytokines produced in response to stimulants and stimulate releasing of other cytokines that act in concert (Jaffer et al., 2010). It is believed that these two cytokines levels are related to poor outcomes from sepsis (Pinsky et al., 1993). Accordingly, endothelial damage, loss of vascular tone, myocardial dysfunction as well as coagulation cascade can lead to reduced blood flow to vital organs such as the kidneys, central nervous system, heart, and eventually death (Caraballo and Jaimes, 2019).

In addition to unregulated systemic inflammation, altered cell metabolism has been suggested as the major disturbance during sepsis (Wasyluk and Zwolak, 2021). It is an important area of interest in terms of sepsis treatment because most studies have focused on inflammatory response control. Sepsis-induced dysfunction and mitochondrial damage are likely the major causatives for disturbing cell metabolism. Mitochondrial damage changes the normal metabolism of cell macronutrients (Wasyluk and Zwolak, 2021). It is important to note that all such biochemical reactions are mediated by producing pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) (Pinsky et al., 1993). The pro-inflammatory cytokines activate catalytic processes that can lead to increased circulating levels of glucose and fatty acids (Wasyluk and Zwolak, 2021). Accordingly, the failure of pyruvate entering into the tricarboxylic cycle leads to increased lactate formation (Wasyluk and Zwolak, 2021). As a result, lactate would accumulate, and metabolic acidosis is expected. Moreover, oxygen usage by sepsis patients is poor, that further contributes to lactate production. Notably, hypoglycemia occurs as sepsis continues (Wasyluk and Zwolak, 2021). Metabolic acidosis may be the primary cause of death in calves with diarrhea or septicemia, induced by gram-negative bacteria (Kasari, 2016). Correcting acidosis should be a primary goal in the treatment of septic calves.

PREVENTIVE STRATEGIES

Prevention of diarrhea or septicemia is the primary goal in controlling calf mortality in dairy farms. Prevention is important from this point of view and severe cases of septicemia can be reduced. Since sick calves are major contaminants of surroundings, decreased number of sick calves would help reduce the shedding of bacteria in the environment (Nikkhah and Alimirzaei 2022b). Prevention of colisepticemia could be addressed from two distinct visages: nutritional or environmental. The importance of colostrum feeding is emphasized in all studies (Tedia, 2012; Bashahum and Amina, 2017). It is necessary to note that high-quality (Brix index > 22 when refractometer is used to determine colostrum quality) and uncontaminated (bacteria count < 100000 cfu/ml) colostrum must be fed in the first 6 hours of life to ensure maximal passive transfer (Phipps et al., 2016; Nikkhah and Alimirzaei, 2022b). On the other hand, colostrum temperature should be checked, especially in cold seasons, and heated in warm water if needed. Low-temperature colostrum (< 37˚C) itself can lead to diarrhea (Vallance and Finlay, 2000). In addition to animal studies, it has been reported in a recent human study that bovine colostrum can be useful in preventing and treating gastrointestinal diseases such as infectious diarrhea (Fasse et al., 2021). As noted above, attachment is an essential process in the pathogenicity of *E. coli*; thus, blocking bacterial attachment is a key factor in preventing bacterial overgrowth. In addition to the role of IgG in local (small intestine) and systemic immunity of calves, colostral IgG has a critical role in preventing bacterial attachment by occupying attachment sites located on the surface of intestinal epithelial cells (Lopez and Heinrichs, 2021).

Millions of microbes are inhabitants in the gastrointestinal tract of mammalians, performing many beneficial biological functions. Immune modulation, nutritional digestion, and protection against pathogenic microbes such as *E. coli* are the most important duties of the gut microbiota (Malmuthuge and Guan, 2016; Zhang et al., 2022). It has been reported that gut microbiota composition and establishment of beneficial bacteria such as *lactobacillus acidophilus or Lactobacillus Plantarum*, *Bifidobacterium bifidum*, and *faecalibacterium prausnitzii* are associated with enteric pathogenic bacteria colonization (Malmuthuge and Guan, 2016). In this regard, using probiotics may be useful in controlling pathogenic bacterial attachment and its subsequent adverse effects. The positive effects of probiotic bacteria in alleviating diarrhea severity has been reviewed (Cangiano et al., 2020). The blockage of bacterial attachment to the intestinal epithelium may provide an opportunity for preventing bacterial overgrowth inside the host. Despite the advantages of diverse gut microbiota, it should be considered that the gut microbiota has a dynamic nature in the early stages of calf life, and many environmental factors affect its composition (Malmuthuge and Guan, 2016). Therefore, modulating the gut microbiota to control enteric infections such as colibacillosis and colisepticemia provides a perspective for research in this critical area. From a pragmatic viewpoint, feeding probiotics in newborn calves' diets is an attempt to optimize the gut environment through the prevention of pathogenic bacterial attachment. The positive effects of including probiotics in calf diets during the early two weeks of life have been reviewed (Cangiano et al., 2020).

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In addition to the nutritional protocols for preventing bacterial overgrowth, other management practices, such as dry cow nutrition and welfare as well as sanitary protocols, can impact calf immune system efficacy and bacterial environmental overload (Cangiano et al., 2020). It has been illustrated that nutritional deficiencies and stress in the dam during late gestation may disturb intestinal function and impair colostral immunoglobulin absorption (Alimirzaei and Nikkhah, 2021). As a result, producers must pay attention to dry cow's comfort to ensure the successful transfer of passive immunity. The pregnant cow must give birth in a comfortable and clean area. It seems that calving in a dirty pen increases the risk of infectious agent shedding by the newborn calf (Nikkhah and Alimirzaei, 2022b). Removing contaminated bedding materials and renewing them after each calving are recommended to reduce pathogenic organisms' transfer from the maternity pen to the calf-rearing area. Furthermore, disinfecting calving pens should be a regular practice on dairy farms. In addition to sanitary protocols, the natural calving process is important in terms of calf health and its performance (Cho et al., 2014). It has been reported that dystocia is closely associated with calf performance as well as susceptibility to diarrhea (Cho et al., 2014). Calves born with dystocia may have a poor ability to absorb colostral IgG, which further predisposes them to colisepticemia. As a result, for a good start, the birth must take place in a clean calving pen under natural parturition. Calf pens, hutches, or barns are considered major centers of pathogenic organisms unless the farm has routine and regular sanitary programs. All facilities and equipment, including bottles, milk containers, colostrum feeding containers, personnel boots, and cloths, must be disinfected after each use.

TREATMENT GUIDELINES

Early identification and treatment of infected calves is the most important practice in blocking *E. coli* prevalance in calf-rearing farms. Sick calves shed the pathogenic agent during the disease period and after recovery (Nikkhah and Alimirzaei, 2022b). Therefore, affected calves must be diagnosed early and treated immediately to reduce the risk of further bacterial shedding. Severe diarrhea, sunken eyes, loss of appetite, recumbency, failure of suckling reflex, and low body temperature, especially in cold seasons, are the most important signs of colibacillosis or colisepticemia in newborn calves (Bashahum and Amina 2017). Treatment of diarrheic or sepsis calves is difficult, especially when the severity of infection is high. Understanding calf physiology during sepsis or diarrhea will help better react to severe cases. Importantly, and as emphasized earlier in this article, farmers must concentrate on decreasing the number of severe cases, because it needs antibacterial or fluid therapy to save affected calves. Treatment of infected calves totally depends on their general health status. According to calf standing ability, four treatment charts have been developed: 1) calf recumbent/unable to stand, 2) calf standing securely, 3) calf stands confidently, enophthalmos, and 4) calf stands confidently with no enophthalmos (Constable et al., 2020). Importantly, in some severe cases of sepsis, it seems that calves need a more therapeutic period with the administration of antibiotics and fluid therapy. As a result, to increase the calf survival chance, treatment protocols in the first diagnosis of sepsis cases are recommended. It is important to note that there is no single antibiotic or fluid therapy protocol on all farms, as it depends on the calf's general health status. For instance, some calves could recover with only using 1 or 2 liters of intravenous therapy; however, in some cases, 8-10 liters of intravenous fluid therapy may be needed. Furthermore, nursery is a critical practice that many dairy farms disregard. Accordingly, calf recovery is a direct function of proper treatment and gentle nursery (Nikkhah and Alimirzaei, 2022a,b; Zhang et al., 2022).

As described, cardiovascular dysfunction, hypotension, and metabolic acidosis are common consequences of sepsis. Administration of anti-inflammatory drugs plus intravenous antibiotics to alleviate inflammatory reactions and eliminate pathogenic *E. coli* are essential in treating sepsis calves. Dexamethasone is an anti-inflammatory drug which can be used successfully in controlling inflammation in sepsis calves. Antibiotics against the infectious agent must be administered intravenously, which could be different from one farm to another (farm observations and experience). Thus, antibiotics should be selected according to the farm's veterinarian protocols. As noted, fluid therapy, both orally or intravenously, is the first line of defense against colisepticemia or colibacillosis. Recumbent and sick calves with suckling inability must be treated intravenously by isotonic dextrose-saline solution to recover electrolytes and glucose loss during sepsis and diarrhea (Nikkhah and Alimirzaei, 2022b). Using hypertonic saline solution (7.2 % NaCl, 2400 mOsm/L) may increase blood pressure, improve oxygenation, and cardiac output, allowing calves to reinstate vital organ functions (Constable et al., 202). As such, providing hypertonic sodium bicarbonate solution (8.2% $NaHCO_3$) may help correct acidosis and return the HCO_3 lost during diarrhea (Wasyluk and Zwolak, 2021). Nursing sepsis calves are vital for monitoring their health status because extra antibacterial or fluid therapy may be needed.

CONCLUSION

Colisepticemia is a threatening neonatal calf disease worldwide with significant mortality rates. Poor dry cow management and welfare, poor colostrum feeding management, unclean calving area, and contaminated calf barns all could predispose newborn calves to diarrhea and septicemia. Early diagnosis through clinical examination of calf general

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health such as standing ability, suckling reflex, abnormally high or low body temperature, and the calf appetite can lead us to suspect colisepticemia. Treatment must be initiated immediately. Effective and innovative treatment of sick calves can be a perfect preventive practice in minimizing bacteria recycling between the calf and its ambiance. Fluid and antibacterial therapy must be the first therapeutic action. Additionally, sick calves need extensive nursing programs because some calves may need extra therapeutic practices such as antibiotics and vitamin injections, and heavy intravenous or oral fluid therapy. As a result, understanding septicemia biology and calf behavior provide pragmatic opportunities for producers to manage infected calves effectively.

DECLARATIONS

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Authors' contribution

The authors contributed equally to this work, including conceptualization, review, strategic contemplation, writing development, editing, and revising. Akbar Nikkhah Led the project. All authors checked and confirmed the final draft of the manuscript.

Competing interests

None.

Ethical considerations

Ethical considerations (e.g., plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been made by the authors.

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Improved Dot-ELISA Assay Using Purified Sheep *Coenurus cerebralis* Antigenic Fractions for the Diagnosis of Zoonotic Coenurosis

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ABSTRACT

Clinicians face significant problems in the diagnosis of zoonotic coenurosis. The current study aimed to develop an improved dot-Enzyme-linked-immunosorbent assay (dot-ELISA) for the diagnosis of zoonotic coenurosis using sheep *Coenurus cerebralis* scolices purified antigen (CcS-Ag) and to compare the obtained results with those of indirect ELISA and Enzyme-linked immunoelectrotransfer blot technique (EITB). Sera were collected from humans and sheep infected or suspected of infection with *Coenurus cerebralis*, control cases, and cases infected with other parasites. The CcS-Ag was proved to be the most specific antigen. This antigen was fractionated, and its specific polypeptides against anti-*C. cerebralis* antibodies (ACc-Ab) were identified using EITB. Fractions at the molecular weight (MW) of 48 and 58 kDa were proved as the only specific ones, eluted from the gel and concentrated, then dotted on the NC sheet as pooled antigen before its evaluation in the diagnosis of infection using dot-ELISA. Dot-ELISA demonstrated absolute 100% sensitivity and 100% specificity as recorded by EITB, compared to both fractions on a nitrocellulose (NC) sheet using surgically proved infected human or sheep sera as a gold standard. Diagnosis by ELISA using crude CcS-Ag revealed similar sensitivity but lower specificity (75%). The diagnostic accuracy of dot-ELISA was proved by comparing its results with postmortem data obtained post slaughtering of 20 suspected sheep and patients investigated by computed tomography (CT) and magnetic resonance imaging (MRI). In conclusion, the selection of specific fractions after EITB to be used in dot-ELISA improved the diagnostic value of the test as a diagnostic tool gathering the benefits of ELISA and EITB.

Keywords: Antigen,*Coenurus cerebralis*, Dot-ELISA, Human, Sheep, Scolices

INTRODUCTION

Clinicians face significant issues when dealing with neurological cases for several reasons, including parasites, which can infect the central nervous system (CNS). Unspecific clinical features associated with the absence of specific diagnostic methods have exaggerated the problem (Varcasia et al., 2022; Yamazawa, 2020). One of these issues is coenurosis, a worldwide zoonotic parasitic infection with the larval stage (*Coenurus*) of *Taenia multiceps*, *T. serialis*, and *T. brauni* that occurs mainly in sheep, goats, horses, cattle, buffaloes, yaks, rodents, rabbits, and humans that act as intermediate hosts (IMH) for the adult worms which develop in the small intestine of some canines, causing taeniasis (Lotfy, 2020). *Taenia multiceps* larvae *(Coenurus cerebralis)* have been thought to be the only ones capable of infecting the CNS, with only one incidence of *C. serialis* infection in the brain found in Iran (Rostami et al., 2013; Yamazawa, 2020). The definitive hosts are infected by eating raw or undercooked tissues infected with *C. cerebralis* cysts, which then develop into adult worms in the small intestine. Afterward, the eggs or gravid proglottids pass into the final host's feces (Ajaj et al., 2021). However, IMH becomes infected after ingesting food or water contaminated with *T. multiceps* eggs or gravid segments of the parasite. In the small intestine, the parasite oncospheres hatch from the eggs and penetrate the intestinal wall. Then, most of the parasites migrate through the blood to the CNS subcutaneously, forming cysts commonly detected in the brain, cerebellum, and subarachnoid spaces (Lescano and Zunt, 2013). Acute or chronic nervous manifestations (headaches, vomiting, ataxia, blindness, and papilloedema) due to intracranial pressure led to death in some cases (Varcasia et al., 2022). These symptoms have been detected in some cases in Egypt described by Antonios and Mina (2000), who found patients infected with *C. cerebralis*. Moreover, *C. cerebralis* causes sheep coenurosis, which is common in Egypt, in which the metacestodes develop in the brain and spinal cord mainly, causing nervous signs, depression, circling, convulsions, head deviation, blindness, and ataxia leading to animals' death and severe economic

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losses (Zhang, 2019). Dogs are a definitive host for *Taenia* species; a high incidence of infection by this metacestode in sheep should be reflected in humans in the same localities (Rostami et al., 2013).

Infection in sheep is diagnosed mainly at Post-Mortem (P.M.) inspection of the suspected cases (Desouky et al., 2011). In contrast, there is no accurate clinical way to differentiate cases of cerebral coenurosis from those of cysticercosis, echinococcosis, or other CNS infection. Moreover, some patients have sterile cysts, making diagnosis challenging with some imaging techniques (Lotfy, 2020). Therefore, the availability of accurate and applicable serological field tests associated with sonography and other clinical-pathological investigations can facilitate the diagnosis.

The diagnostic assay ELISA is simple to use and can evaluate numerous samples simultaneously. However, due to a high percentage of false-negative results and occasional cross-reactions with other parasite disorders, its sensitivity and specificity are influenced by the degree of purity and specificity of the used antigens (Sun et al., 2015). While more sensitive diagnostic methods, such as Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) antigen fractionation and Western blot or enzyme-linked immunoelectrotransfer blot (EITB) fractionation can be applied, they are not considered to be field tests (Sabry, 2007; Mahdy et al., 2017).

Dot-ELISA is a special modification for ELISA assay. It has more advantages than the indirect ELISA as it can be performed using a minute volume of reagents and is easily read visually. For this reason, dot-ELISA is considered an accurate test, gathering the benefits of both ELISA and EITB (Taher et al., 2017).

Due to the previous explanations, this study aimed to create an improved dot-ELISA assay for diagnosing human and animal coenurosis, which was performed by using specific protein fractions extracted from sheep *C. cerbralis*. Regarding positive and negative sheep and patient sera, the sensitivity and specificity of the assay were evaluated and compared to those of indirect ELISA and EITB techniques. Comparing the test results with the P.M. data of suspected sheep was also considered.

MATERIALS AND METHODS

Ethical approval

This research was carried out per the principles of the Helsinki Declaration. The procedure was accepted by the Faculty of Medicine, Cairo University's Scientific Research Ethics Committee (Vet CU20022020132). All Patients enrolled in the study were informed verbally about the aim of the study and the future examination that would be applied to their collected stool and blood samples. The institutional review board approved the procedures for handling and sample collections from animals of the Institutional Animal Care and Use Committee (IACUC) of Cairo University, Egypt. The research took place at Cairo University's Department of Parasitology in the Faculty of Veterinary Medicine from September 2021 to March 2022.

Collection and identification of cysts

Symptomatic Coenurusosis suspected 1-year-old Baladi sheep males of 60± 5.5 kg weight, showing nervous manifestations (depression, circling, head deviation, blindness, and ataxia) were gathered from private sheep farms at Fayoum Governorate, Egypt. Then, they were sent for Halal slaughtering at Fayoum slaughterhouse, Fayoum Governorate, Egypt. After that, transverse sectioning of their heads was carried out during the P.M. examination. Then, bladder-like cysts present in the cerebrum and cerebellum of their brains were collected (Figure 1). The collected cysts were transferred in an icebox to the Department of Parasitology, Faculty of Veterinary Medicine, Cairo University, for further examinations and antigen preparation. The collected cysts were examined parasitologically and identified according to Desouky et al. (2011). Identified blood samples of the inspected infected sheep were also collected from the jugular vein into a 10-ml centrifuge tube without anticoagulant (Abdel-Rahman and Abdel-Radi, 2022).

Preparation of *Coenurus cerebralis* antigens

Cysts were collected from all positively infected sheep which proved after P.M. inspection, but only fresh extracted non-calcified cysts with numerous macroscopic scolices, intact transparent wall, and clear cystic fluid were used for antigens preparation as follows:

Coenurus cerebralis crude fluid antigens

Coenurus cerebralis crude fluid antigens (CcF-Ag) were prepared as described by Jeyathilakan et al. (2021) with slight modification. Briefly, after washing the cyst using 0.01 M sterile phosphate buffer saline (PBS), with a pH of 7.4, its fluid was aspirated using a needle. The fluid was centrifuged at 8,000 g for 30 minutes by using IEC Centra CL2 General-purpose benchtop centrifuge (United States). Then the supernatant was collected, and 0.02% sodium azide was added. It was dialyzed for 24 h in the refrigerator using a dialysis membrane (6000-8000 MW cut-off) (Sigma, USA) against 5mM Tris–HCl (pH 7.4). The fluid was then concentrated versus poly-ethylene glycol 6000 (SRL, China). Using

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the Bradford method (Bradford 1976), its content was measured, allocated into a 1.0 ml Eppendorf tube, and kept at -20 °C.

Coenurus cerebralis scolices crude antigens

Coenurus cerebralis scolices crude antigens (CcS-Ag) were prepared as previously described by Hassanain et al. (2016) with few modifications. By dissecting the scolices neck away from the cyst wall with sterile fine forceps and scissors, the scolices were removed from the cyst wall. They were collected in a sterile tube and washed with PBS (pH 7.4) three times. The tissue was suspended in ten volumes of sterile PBS. The mixture was exposed to three cycles of freezing and thawing. Then, the contents were homogenized using a homogenizer (ULTRA- TURRAX Janke and Kunkel KG) for 15 minutes in an ice bath. The content was sonicated using Cole Parmer ultrasonic homogenizer for 5 minutes (under 150-watt interrupted pulse output with 10-second bursts and five seconds intervals). The mixture was centrifuged at 10,000 g at 4 °C for 30 min. After adding 0.02% sodium azide to the collected supernatant, it was dialyzed, and its protein contents were measured, allocated, and stored at -20°C.

Coenurus cerebralis wall crude antigens

As previously described, for the preparation of the scolices antigens, a clean, sterile cyst wall was washed with PBS (pH 7.4) and then dissected into small pieces using sharp forceps and scissors. The pieces were suspended in a ten-time volume of PBS and then exposed to freezing and thawing three times. The mixture was homogenized, then sonicated and processed as in the case of scolices antigen preparation. The supernatant was collected, and its protein contents were measured, allocated, and stored at -20°C.

Selected sera

Sheep blood samples were collected from the jugular vein into 10 ml tube without anticoagulant. Using venipuncture, human blood samples were collected from a superficial vein in the upper limb In addition, serum samples were prepared and then stored at -20°C to be used in the study.

Human sera

A total of 85 human serum samples were included in the study. Five serum samples from surgically proved *C. cerebralis* infected patients in the first group (G-1, 30-45 years-old males), 20 serum samples from suspected *C. cerebralis* infected people in the second group (G-2, 32-50 years-old males) at high risk of coenurosis infection, who worked in shelters to collect stray dogs, and went to private neurology clinics, tropical medicine outpatient clinics, Cairo University Hospitals, and Fayoum University Hospital. Those people complained of some neurological symptoms, including chronic headaches, nausea, weakness, weight loss, disturbances of personality, visual disturbance, drowsiness, facial palsy, paraesthesia, ataxia, hemiparesis, and sensory impairment with or without shadows in Computed tomography (CT) of the brain.

Forty serum samples in group 3 (G-3) were used to assess cross-reactivity in the study which were collected from patients with known infection with other parasites previously confirmed by stool, blood smears, and other specific tests such as serological tests (ELISA and IHA). These serum samples included 20 infected by *Schistosoma mansoni* (*S. mansoni*), ten harbor *Taenia* eggs in the stool, and ten surgically proved that they were infected with *Hydatid* cysts (Hc) in their lungs. A total of 20 serum samples from healthy individuals were collected in fourth group (G-4).

Sheep sera

To analyze the sheep, 10 serum samples were collected from *C. cerebralis* infected sheep proved during the P.M. examination of their heads and were selected as a positive control (G-1). The second group (G-2) included 20 samples from suspected *C. cerebralis* infected sheep suffering from clinical signs of coenurosis (depression, circling, head deviation, blindness, and ataxia), and the authors could inspect these animals post slaughtering, were selected to evaluate the sensitivity of the diagnostic technique. For the third group (G-3), 50 serum samples were used to assess cross-reactivity of *C. cerebralis* tested antigens. The samples were collected from sheep with known infection by other parasites previously confirmed by stool, blood smears, and other specific tests. These serum samples included 20 infected by *Cysticercus ovis* (*C. ovis*), ten harbored *Hydatid* cysts, and 20 infected by *C. tenuicollis*. Finally, the fourth group (G-4) entailed 20 samples from healthy non-infected sheep, which were used as a negative control.

Indirect ELISA

Briefly, the assay was performed as described by Liu et al. (2015) with few modifications. Following checkerboard titration, 200 µl/well of each antigen (4 mg/ml) were separately added to the 96-well Poly-ethylene ELISA plates in carbonate buffer (pH 9.6), and the plates were then incubated for 1 hour at 37 °C. The wells were washed three times in washing buffer (0.5% Tween 20 in PBS pH 7.3 0.2), and then blocked with 200 ml of PBS-0.05% Tween 20 (PBST) containing 0.5% bovine serum albumin (BSA) and incubated as before. Following washing, the following reagents were subsequently added (100 ml/well), and each step was incubated at 37°C for 1 hour, firstly tested sera at a 1:100 dilution in PBST, and then horseradish peroxidize HRP-conjugated anti-sheep IgG (Sigma, USA) diluted 1:2500.

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After adding O-phenylene diamine dihydrochloride (OPD, Sigma, USA) as a substrate and 100 ml/well H2O2, the reaction was observed, then it was stopped by adding 1 N H2SO4 (50 ml/well). Optical density (OD) values were measured at 450 nm using a microplate reader (Titerteck multiskan ELISA reader). Serum samples were tested in duplicate reference; known infected and non-infected sera were associated with each plate. The cut-off values were calculated as double the mean negative controls (Lardeux et al., 2016). Mean standard deviation, sensitivity and specificity were calculated statically.

This assay was used for two purposes; the first was a selection of the best crude larval antigens (CcF-Ag, CcS-Ag, and *C. cerebralis* wall crude antigens [CcW-Ag]) in capturing their specific anti-Cc antibodies (ACc- Ab) and exclusion of non-specific antibodies present in sera infected with other parasites. The second purpose was to evaluate this assay in diagnosing coenurus infection using these selected antigens compared with the other improved dot-ELISA assays.

Fractionation of *Coenurus cerebralis* crude antigen and transferring of protein

Under reduced circumstances, SDS-PAGE analysis of CcS-Ag was performed using a 12 % non-gradient slab gel and a 5 percent stacking gel (Laemmli, 1970). A 20 mA current was used to fractionate the antigen. The gel was calibrated using standard markers and the molecular weight (MW) (Sigma SDS-100B). According to a previous report by Towbin et al. (1979), CcS-Ag fractionated proteins were transferred from the gel onto nitrocellulose paper (NC) overnight at 10 V, 100 mA, and 4 C. Drying and storing the NC sheet at -20 C.

Determination of *Coenurus cerebralis* specific protein fractions using Enzyme-linked immunoelectrotransfer blot technique

Longitudinal NC strips (15X 0.5 cm) representing the fractionated antigen were cut out. The strips were incubated with 3% BSA at 37 °C for 1 hour to block the non-specific antibody binding sites. According to Towbin et al. (1979), the strips were washed in PBS and then incubated with 2.0 ml of serum/strip and a 1:100 dilution of known positive and negative control serum samples for 2 hours using EITB. After being washed, each strip was treated in a blocking buffer containing HRP-conjugated anti-human or anti-sheep IgG (Sigma, USA) at a dilution of 1:1.000 for 1 hour at 37°C. While being continuously observed, the addition of peroxidase substrate (4-chloro-1-naphthol, Sigma) revealed the interaction between IgG and the antigen.

Nitrocellulose paper strips were reacted against positive and negative sera from cases with known infection history to detect *C. cerebralis*-specific diagnostic protein fractions. The specific fractions are those reacting positively with *C. cerebralis* infected cases and did not react with negative control or those infected with other parasites. To identify the specific protein bands throughout the entire NC sheet in relation to the MW standard, these strips were retained in their original positions on the NC sheet. Moreover, the identified specific fractions were used to identify infection in all tested serum samples by EITB as a diagnostic technique to be compared with the accuracy of dot-ELISA.

Elution and concentration of the specific protein bands from the gel

Elution and concentration of the specific protein fractions from the gel were performed as described by Taher et al. (2017). In brief, longitudinal strips containing the MW standards and the outermost portion of the fractionated antigen were cut out following the SDS-PAGE analysis of Cc-Ag. Tsai and Frasch (1982) reported that coomassie blue was used to dye the strips. The proteins with MWs of 48 kDa and 58 kDa were identified and transverse gel strips corresponding to those proteins were cut out horizontally across the whole gel. This gel strip was moved to an elution tube with a membrane connecting it (6-8 MW cut-off) from Spectrum Medical Inc. in Los Angeles, California, and filled with PBS (pH 7.4). The process was carried out overnight at 10 V, 100 mA under 4 °C with the tubes stored in a Bio-Rad elution unit. According to Bien et al. (2013), 0.02% sodium azide was added to the collected solution after the gel material was removed, resulting in a smaller amount of solution, compared to poly-ethylene-glycol in the molecular porous membrane tubing (6-8 MW cut-off). Five elution runs were performed after cutting the gel slice containing the kDa from thick (1 ml thick) gel to obtain enough amount of this specific fraction to perform the study. The protein content of the concentrated eluted material was calculated and placed in a 250 MM tube, which was then stored at -70°C.

Dot-ELISA technique

The technique was carried out as described by Taher et al. (2017) with slight modification using a piece of NC described by Boctor et al. (1987). The eluted concentrated pooled protein fractions with MWs of 48 and 58 kDa were dot-plotted onto a 10X 10 cm NC sheet that had been separated into 10X 10 mm squares using a pencil. The sheet was then

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maintained in an appropriate Petri dish. Tests on Sera were conducted twice. The sheet was immersed in 3.0% BSA to block the non-specific binding sites. After being washed three times by PBS-T, they were allowed to dry once more before 3 µL of the tested and control sera (1: 100 dilution in duplicate) were spotted on each dot. They were then allowed to dry for 15 minutes before being washed once more by PBS-T. Then, each square received 3 µL from 1:1000 HRP-conjugated anti-Human or anti-sheep IgG (Sigma, USA) and was dotted with the solution before being allowed to dry. After being washed three times, the sheet in the dish was immersed in 5 ml of the substrate solution (4-chloro-1-naphthol, 340 mg/ml substrate buffer, and 0.03 percent hydrogen peroxide solution). The discs were observed; typically, the color changed after 10 to 15 minutes. A well-defined blue-purple spot was considered positive and evaluated in comparison to the reference control and tested sera after the dot's color changed, which was visible with the naked eye (Figure 2). Additionally, the sensitivity and specificity were computed.

Statistical analysis

The statistical program SPSS, version 28, was used to code and enter the data for this investigation (IBM Corp., Armonk, NY, USA). Mean, standard deviation, median, minimum, and maximum were used to describe quantitative data, while frequency (count) and relative frequency were used to do so for categorical data (percentage). To compare numerical variables, the non-parametric Mann-Whitney test was applied (Chan, 2003a). Using the Chi-square (c^2) test, categorical data were compared. When the anticipated frequency was less than 5, the exact test was utilized (Chan, 2003b). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic efficacy of common diagnostic indices were calculated as indicated by (Galen, 1980). Area under curve (AUC) analysis was used to construct the Receiver operating characteristic **(**ROC) curve and find the optimal ELISA cut-off value for infection detection. A P-value of less than 0.05 was regarded as statistical significance

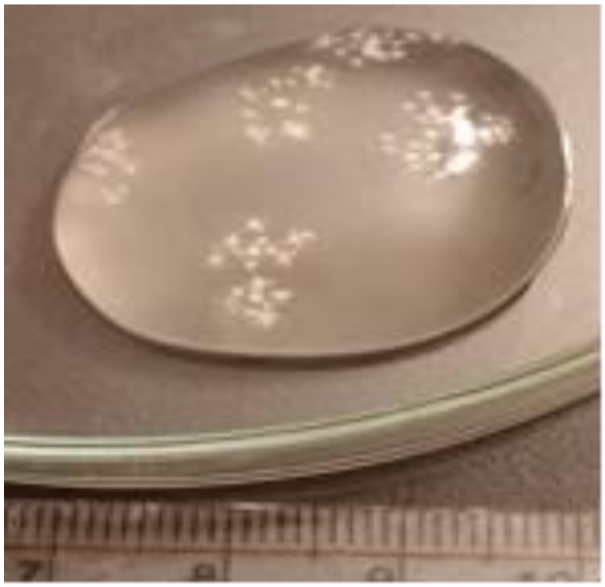

Figure 1. *C. cerebralis* bladder-like cyst freshly extracted from Baladi sheep brain (about 30 mm in diameter)

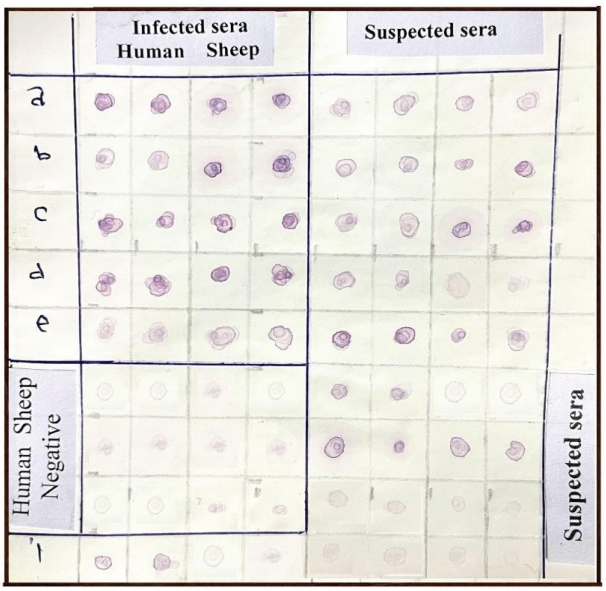

Figure 2. Improved Dot-ELISA on a nitrocellulose sheet using specific CcS-Ag protein fractions versus surgical proved infected patients, infected Baladi sheep sera, negative control, and some suspected sera.

RESULTS

Selection of the most diagnostic *Coenurus cerebralis* crude antigen

The data in Table 1 revealed that CcF-Ag and CcS-Ag showed absolute (100%) sensitivity in the diagnosis of anti-Cc antibodies (ACc-Ab) in sera of infected sheep in comparison with CcW-Ag that failed in capturing specific Ab in two positive serum samples of infected sheep that were proved to be infected after P.M. inspection of their brains. CcS-Ag had high mean specificity than the other two Ag reaching 88.57%, while it was 80% and 74.28% for CcF-Ag and CcW-Ag respectively. Both CcF-Ag and CcS-Ag demonstrated the same specificity in the diagnosis of anti-*C. ovis* Ab in infected sheep while CcW-Ag showed a high level of cross-reaction versus anti-*Hc* -Ab and anti-*C. tenuicollis* Ab in infected sheep sera. These data proved that CcS-Ag could be used as a diagnostic Ag more accurately than the other *C. cerebralis* tested Ag.

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Table 1. Sensitivity and specificity of *Coenurus cerebralis* crude antigen in the diagnosis of infection in Baladi sheep using indirect ELISA

	Tested sera collected from		Reaction of tested sera versus C. *cerebralis*					
			Fluid Ag		Scolices Ag		Wall Ag	
	sheep infected by	No. exam	No. +Ve	Percentage	No. +Ve	Percentage	No. + Ve	Percentage
Sensitivity	*C. cerebralis*	10	10	100	10	100	8	80
Specificity	*C. ovis*	20	2	90%	2	90%	3	85
	Hydatid cyst	10	5	50	3	70	6	40
	C. tenuicollis	20	5	75	3	85	5	75
	Non infected sheep	20	2	90	0	100	4	80
	Total Mean specificity	70	14	80%	8	88.57	18	74.28

No. exam: Number of examined cases, *C. cerebralis*: *Coenurus cerebralis*, Ag: antigen

Determination of *Coenurus cerebralis* reacted polypeptides in scolex crude antigen using Enzyme-linked immunoelectrotransfer blot technique

The treatment of NC strips carrying the fractionated CcS-Ag of sheep origin against infected and controlled sheep and human sera using EITB revealed several specific and cross-reacted fractions (Figure 3). Seven reactive bands, corresponding to MW standard at 18,22, 26-34, 48, 58, 75, and 112 kDa were recognized compared to control infected sheep sera (Figure 3, Lane 1).

Nine bands reacted positively after the treatment by *C. cerebralis* of similar strips from surgically approved infected patients at MW of 18, 22, 26-34, 48, 58, 63, 75, and 98 kDa (Figure 3, Lane 2).

The fractions at MW of 18, 22, 29, 38, 63, 75, 98, and 112 kDa were cross-reacted against Hydatid cyst (H. cyst) infected patient sera (Figure 3, Lane 3). The fractions at kDa 22, 24, 29, 63, and 75 kDa were also reacted against *S. mansoni* infected patient sera (Figure 3, Lane 4), while the fractions at MW 18, 22-24, 35 and 63 kDa (Figure 3, Lane 5) were cross-reacted against sera of *Taenia* spp. infected patients. At the same time, fractions with MW of 18, 24, and 63 kDa were falsely reacted against intact sera (Figure 3, Lane 6), and fractions with MW of 11-15, 18-22, and 34-36 kDa were falsely reacted against healthy sheep sera (Figure 3, Lane 7).

Among all the previous fractions, the two fractions at MW 48 and 58 kDa in strips 1 and 2 were considered *C. cerebralis-specific* fractions. These fractions were recognized only in sheep and humans surgically approved *as C. cerebralis* (gold standard) infected sera and not recognized in sera of patients infected with other parasites, as well as in human and sheep negative control sera (Figure 3).

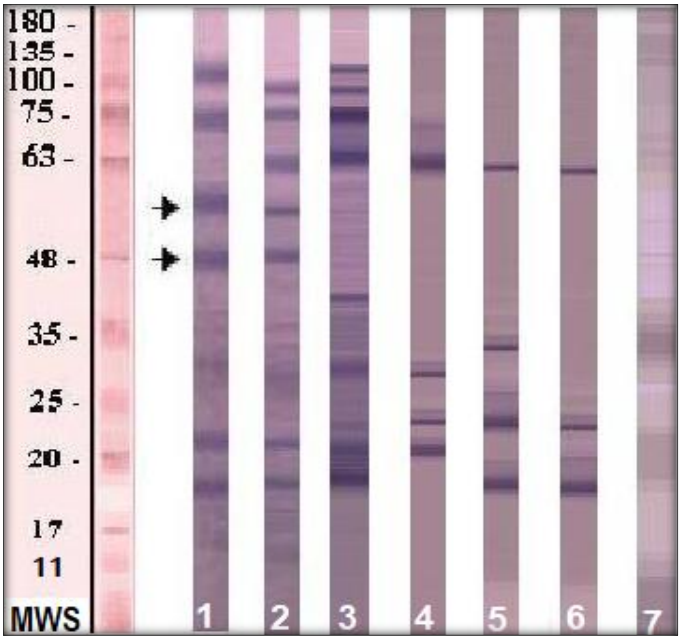

Figure 3. Reacted polypeptides in fractionated CcS-Ag of Baladi sheep on NC strips treated with infected and control sera using EITB. Treated versus *C. cerebralis* infected sheep sera (Lane 1), treated by *C. cerebralis* infected patients' sera (Lane 2), treated by *Hydatid* cyst infected patients sera (Lane 3), treated versus *S. mansoni* infected patients sera (Lane 4), treated by *Taenia* spp. infected patients sera (Lane 5). Treated by healthy control in human sera (Lane 6), control non-infected sheep sera (Lane 7). MWst.: Molecular weight protein slandered (Sigma).

Efficacy of indirect, Dot-ELISA, and EITB for the diagnosis of infection

Comparing the efficacy of indirect ELISA using crude CcS-Ag and dot-ELISA that was performed using pooled eluted concentration fractions (MW 48 and 58 KDa) in the diagnosis of infection, the data revealed that both assays showed absolute (100%) sensitivity in the diagnosis of ACc-Ab in sera of surgically proven infected patients and sheep (Table 2). Concerning the test specificity, indirect ELISA showed low specificity in the diagnosis of antibodies of other parasites present in sera from patients infected with H. cyst, *S. mansoni*, and *Taenia* spp. eggs like 40%, 25%, and 30% from the sera infected by the previous parasites, respectively. They reacted as false positive against the used CcS-Ag. This cross-reaction decreased the test's specificity to 60%, 75%, and 70% in these patients' sera. These false-positive cases' mean OD values were 0.463 ± 0.020, 0.420 ± 0.019, and 0.375± 0.041, respectively. At the same time, 15% of the healthy people demonstrated false reactions against this crude antigen with a low mean OD value (0.403 ± 0.034) as well

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as four sheep from the control non-infected group also reacted as false positive with a mean OD value of 0.460 ± 0.053 (Table 2).

On the contrary, screening of the same previous sera using EITB or dot-ELISA did not show any false-positive reactions against the control negative or other sera as they revealed absolute (100%) sensitivity and specificity using the previous two specific fractions (MW 48 and 58 KDa). Using EITB, these two fractions demonstrated significant reactions during the treatment of NC strips against control infected samples. In contrast, it did not react against healthy control. Moreover, they did not cross-react with antibodies in patients' sera infected with H. cysts, *S. mansoni*, or *Taenia* spp. Using dot-ELISA to diagnose anti-Cc-Ab in the previous sera revealed consistent results with the previously recorded EITB, as both showed 100% specificity corresponding to 75% only for screening the same sera against CcS-Ag by indirect ELISA (Table 2).

Table 2. Difference in diagnostic sensitivity and specificity between indirect and Dot-ELISA assay

Tested human and sheep infected and control sera		No. exam.		Diagnosis by ELISA versus Crude CcS-Ag			Using EITB or dot-ELISA	
				No. + Ve	SP (%)	ELISA OD (Mean ± SD.)	No. + Ve	SP (%)
Human	*C. cerebralis*	5	TP	5	100	0.660 ± 0.093	5	100
			FN	0			0	
	H. cyst	10	TN	6	60%	0.143 ± 0.023	0	100
			FP	4		0.463 ± 0.020	0	
	S. mansoni	20	TN	15	75%	0.159 ± 0.015	0	100
			FP	5		0.420 ± 0.019	0	
	Taenia spp.	10	TN	7	70%	0.165 ± 0.022	0	100
			FP	3		0.375 ± 0.041	0	
	Healthy control	20	TN	17	85%	0.148 ± 0.023	0	100
			FP	3		0.403 ± 0.034	0	
	Mean specificity	60	TN	45	75%		0	100
			FP	15			0	
Sheep	*C. cerebralis*	10	TP	10	100	0.708 ± 0.076	10	100
			FP	0			0	
	Healthy control	20	TN	16	80%	0.123 ± 0.006	0	100
			FP	4		0.460 ± 0.053	0	

No. exam: Number of examined cases. TP: True Positive, FP: false Positive, TN: True negative, FN: false negative, SP: Specificity, OD: Optical density, SD: Standard deviation.

Efficacy of indirect, Dot-ELISA, and EITB for the diagnosis of infection in suspected cases

Investigating the efficacy of the used assays in the diagnoses of infection in 20 symptomatic *C. cerebralis* suspected patients and sheep, the results obtained after EITB was compatible with that of dot-ELISA. At the same time, both tests showed high sensitivity than indirect ELISA (Table 3). Indirect ELISA showed a false diagnosis of infection in 11 patients from 20 suspected (55%). Screening the same patients' sera using EITB or Dot-ELISA revealed infection in 4 cases (20%). Further investigation of these 4 cases using CT and MRI inspection proved that they had cysts in their brain.

For the sensitivity of the used assays in the diagnoses of infection in 20 suspected sheep isolated from private farms with the coenurosis characteristic symptoms, indirect ELISA was used to diagnose the parasite-specific antibodies in 12 animals (60%). A re-examination of this group of sheep using EITB or by the improved dot-ELISA identified another three more positive sheep as infected by the parasite which confirmed by detection of *C. cerebralis* cysts in their brain of them at their inspection post slaughtering (Table 3).

The performance of both types of ELISA in the diagnosis of coenurosis in suspected patients and sheep was statistically evaluated. Receiver operating characteristic **(**ROC) curve was developed for the results of tested serum samples of positive cases, with confirmed coenurosis-infected cases proved after surgical removal or P.M. inspected sheep (Figure 4 and Table 4). The statistics for ELISA results are described in Table 5.

For all positive cases diagnosed by EITB or dot-ELISA were proved to have parasite cysts, the sensitivity and specificity of both tests reached 100%, while as described in Table 5, the sensitivity and specificity of ELISA in the suspected patients were 100% and 72.22%, respectively. In addition, it was 80% and 100% in the suspected sheep, respectively. At the same time, the PPV of the test was very low in patients than in sheep, while the NPV was considered high in patients than in symptomatic sheep. The test reported 75.00% and 85.00% accuracy in patients and sheep, respectively (Table 6).

The data in Table 7 revealed direct relation between the mean value of ELISA OD and the strong positive color determined using dot-ELISA visual reading. The mean of the OD duplicates was used to calculate the Area under the Curve (AUC), which represents the test's accuracy. In the case of suspected patients, the AUC was 0.976, whereas, in the case of suspected sheep, it was 0.987. The best cut-off points, determined after ROC analysis, were 0.403 and 0.157 for

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the OD values in both cases. These values showed ELISA sensitivity and specificity of 100% and 94.4%, respectively. In the case of suspected patients, they were 93% and 100% in cases of suspected sheep. Inspection of the relation between mean ELISA OD and visuals values of Dot-ELISA revealed a significant relation between the degree of darkness of the fraction in EITB or the dot on the NC sheet and the mean OD value of indirect ELISA. This was observed in *C. cerebralis* surgically proved infected cases in humans and sheep. After screening by dot-ELISA, false-positive cases in non-infected controls with low ELISA OD values appeared negative. Also, in the patients infected with other parasites (four by H. cyst, five by *S. mansoni* and three by *Taenia* spp. eggs), they previously demonstrated moderate to low positive ELISA OD values but were negative when screened with dot-ELISA.

Table 3. Diagnostic efficacy of improved dot- ELISA in comparison with indirect ELISA for detection of the infection in suspected cases

	No. exam.		Diagnosis by indirect ELISA versus Crude CcS-Ag			Using EITB or dot- ELISA	
			No.	Percentage	ELISA OD (Mean ± SD.)	No.	Percentage
Suspected people	20	+ Ve	11	55	0.434 ± 0.018	4	20%
		- Ve	9	45	0.148 ± 0.020	16	80%
Suspected sheep	20	+ Ve	12	60	0.469 ± 0.175	15	75%
		- Ve	8	40	0.101 ± 0.040	5	25%

No. exam: Number of examined cases, SD: Standard deviation, OD: Optical density, CcS-Ag: *Coenurus cerebralis* scolex antigen.

Table 4. ROC curve values for prediction of infection using indirect ELISA

	AUC	P value	95% Confidence Interval		Cut off value	Sensitivity (%)	Specificity (%)
			Lower Bound	Upper Bound			
Patients	0.976	< 0.001	0.931	1.021	0.403	100	94.4%
Sheep	0.987	< 0.001	0.946	1.028	0.157	93.3	100

Table 5. Value of ELISA in differentiation between positive and negative suspected samples

	Dot-ELISA						
	Positive			Negative			P value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Patients	0.439	0.408	0.451	0.163	0.116	0.142	0.001
Sheep	0.515	0.141	0.666	0.088	0.058	0.152	< 0.001

Table 6. Accuracy of indirect ELISA in the prediction of infection

Indices	Suspected patients		Suspected sheep	
	Value (%)	95% CI	Value (%)	95% CI
Sensitivity	100.00	39.76 to 100.00	80.00	51.91 to 95.67
Specificity	72.22	54.81 to 85.80	100.00	47.82 to 100.00
PPV	28.57	19.11 to 40.38	100.00	
NPV	100.00		62.50	37.72 to 82.10
Accuracy	75.00	58.80 to 87.31	85.00	62.11 to 96.79

PPV: Positive predictive value, NPV**:** Negative predictive value, CI: Confidence interval.

Table 7. Relation between positive ELISA value and positive dot- ELISA reading

	Value of mean indirect ELISA OD of positive serum samples							Level of positive dot-ELISA				
	No. of positive samples in infected cases	High OD. (~ 0.6)		Moderate OD (~ 0.45)		Low OD. (~ 0.3)		No.+Ve	Strong positive		Weak positive	
		No.	%	No.	%	No.	%		No.	%	No.	%
Human	*C. cerebralis* (5/5)	5	100					5	5	100	-	-
	Suspected (11/20)	2	18.18	4	36.36	5	45.45	4	2	50	2	50
	H. cyst (4/10)	2	50	1	25	1	25	0	-	-	-	-
	S. mansoni (5/20)	-	-	3	60	2	40	0	-	-	-	-
	Taenia spp. (3/10)	-	-	3	100	-	-	0	-	-	-	-
	Control (3/20)	-	-	-	-	3	100	0	-	-	-	-
Sheep	*C. cerebralis* infected (10/10)	6	60	4	40	-	-	10	10	100	-	-
	Suspected (12/20)	8	66.6	4	33.3	-	-	15	8	53.33	7	46.66
	Control (4/20)	-	-	-	-	4	100	0	-	-	-	-

No: Number; OD: Optical density; *C. cerebralis: Coenurus cerebralis*; *S. mansoni*: *Schistosoma mansoni.*

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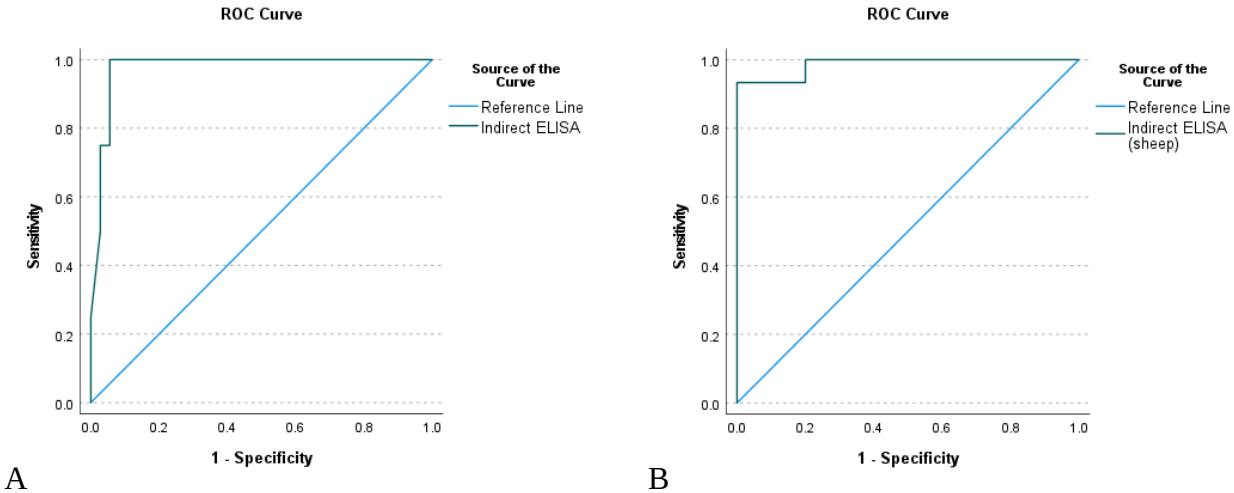

Figure 4. Receiver operating characteristic (Roc) curve for prediction of infection using indirect ELISA. A: Suspected patients, B: Suspected Baladi sheep

DISCUSSION

Coenurosis infection in people or animals is symptomatically and clinically misdiagnosed with several other CNS infections, such as metastatic brain tumor, glioblastoma, brain abscess, primary CNS lymphoma, and neurocysticercosis caused by *T. solium*. The CT, MRI, and sonography with the symptoms and clinical data are beneficial in reaching an accurate diagnosis. Tests, such as MRI, CT, and sonography, are expensive, time-consuming, and considered less sensitive, especially for diagnosing early infection. Therefore, an accurate diagnosis was performed by postmortem (P.M.) examination in animals and surgical removal in humans (Lotfym, 2020; Yamazawa et al., 2020).

The availability of sensitive serological tests that can diagnose the specific Anti-Cc-Ab in sera could be an extra tool supporting the other diagnostic methods. However, these techniques (ELISA, EITB) face other problems, such as selecting a filed applicable test and a source for the specific diagnostic antigen that can capture the characteristic Cc-Ab in infected cases. The present study investigated the importance of *C. cerebralis* antigens extracted from cysts of sheep origin to be used to diagnose infection in patients.

ELISA is one of the most common field serological tests that are easily applied and valuable for simultaneously examining a large number of samples. ELISA specificity is usually affected by the degree of purity and specificity of the antigens (Sabry, 2007; Taher et al., 2017). EITB technique is considered a highly specific diagnostic technique, but it is unsuitable for field applications (Mahdy et al., 2017; Ramadan et al., 2021). The identification of specific diagnostic protein fractions then elution and concentration of this fraction to use in modified ELISA technique after dotting a minute amount of this purified antigen on NC sheet or even in micro-ELISA technique introduce more specific diagnostic assay described as dot-ELISA. This technique was first described by Boctor et al. (1987) for diagnosing schistosomiasis in Egypt. Then, it was modified by Taher et al. (2017) and successfully applied in diagnosing trichinosis infection. Later, Mahdy et al. (2020) used it in hydatidosis diagnosis. Dot-ELISA still has the advantage of the original assay regarding its simplicity, non-time consuming, low cost, and easy investigation of large samples in a short time. Moreover, the test is superior to the original ELISA assay as it can be performed using a minute volume of reagents, serum, and antigens can be evaluated visually without special readers. So, Dot-ELISA is considered a specific test, gathering the benefits of both ELISA and EITB (Taher et al., 2017).

Three types of antigens can be extracted from fresh *C. cerebralis* cysts, including fluid antigen (CcF-Ag), scolices antigens (CcS-Ag), and cyst wall (CcW-Ag). Scolices and fluid antigens are the most commonly used diagnostic antigens in similar metacestodes (Mahdy et al., 2017). The first part of this study used indirect ELISA to test the diagnostic specificity and sensitivity of these crude antigens in capturing anti-Cc-Ab in sera of surgically proven *C. cerebralis* infected sheep, control non-infected sheep, and sera collected from sheep infected by other parasites in order to identify the most diagnostic *C. cerebralis* antigen. CcF-Ag and CcS-Ag proved to have higher diagnostic sensitivity and specificity than CcW-Ag (Huang et al., 2016; Mahdy et al., 207; Ramadan et al., 2021). In contrast, CcS-Ag proved to have higher diagnostic specificity than the other two antigens. The superiority of scolices antigens over fluid antigens in this subject disagreed with Carmena et al. (2006). They described fluid antigens of the bladder worms as specific and high protein content than other antigens. The selection of scolices antigen in this field was supported by Zhang et al. (2012) and Ramadan et al. (2021). As they mentioned, fluid antigens of metacestodes usually contain some proteins from the

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hosts, such as IgG, that might interfere with the accuracy of diagnosis because it cross-reacted with the specific immunoglobulin present in infected host sera.

The identification of the polypeptide profile of this scolices antigen versus known infected and control sera using EITB revealed seven and nine reacted bends versus infected sheep and patients' sera. These fractions in the MW range of 18, 22, 26-34, 48, 58, 75, and 112 kDa were versus infected sheep sera and corresponded to MW of 18, 22, 26-34, 48, 58, 63, 75, and 98 kDa versus control infected patient sera. Most of these fractions cross-reacted with one or more of the tested serum groups from non-infected patients or those infected by H. cyst, *S. mansoni*, or *Taenia* spp. infected patients. The only two fractions at MW 48 and 58 kDa were *C. cerebralis* specific fractions, as they were recognized only by sheep and human sera infected by *C. cerebralis*.

Most of the previously recorded cross-reacted protein fractions with Ab of other bladder worms except those of 48-58 kDa were previously mentioned by Jeyathilakan et al. (2021). They identified the fractions at MW of 12, 28, 42, 52, 98, and 112 kDa in fractionated CcS-Ag of sheep origin that were cross-reacted versus Ab present in experimentally produced anti-hydatid cysts and Ab present in experimentally produced hyper-immune sera (HIS) after immunization of rabbits by Hc scolices antigens. This high number of cross-reacted fractions might be related to the nature of the tested experimentally produced HIS, not versus naturally infected sera as in the present study. The first part of this work succeeded in identifying CcS-Ag and the two fractions at MW of 48 and 58 KDa as specific crude and purified antigens, respectively. The study then used EITB and dot-ELISA to examine the efficacy of indirect ELISA utilizing crude Ag or the value of specific fractions in diagnosing infection in various known infected and control sera.

Using indirect ELISA to diagnose anti-Cc-Ab in tested sera revealed absolute sensitivity for this test with high ELISA OD values. Still, cross-reaction with antibodies of other parasites was recorded, decreasing the mean specificity of the test to 75% with low OD values in all the cross-reacted cases. The recorded absolute sensitivity could be attributed to the source of this control sera as they were from patients or animals that were surgically proved to be infected. This IgG Ab was developed during prolonged migration of the parasite and its survival as an active infection. However, the test revealed several false-positive reactions in control healthy samples. This result agreed with Sun et al. (2015) and Taher et al. (2017) because using crude antigens in this technique adversely affected its specificity for excluding other cross-reacted parasitic Ab as that of hydatidosis, schistosomiasis, and taeniasis in the present study. The authors agreed with Jeyathilakan *et al.* (2021), who mentioned that selecting specific antigens was important for developing specific diagnostic assays and overcoming the indirect ELISA disadvantages concerning the specification of the used antigens.

In comparison to the previously detected specific KDa at MW of 48 and 58 KDa that were previously diagnosed by reference control sera, the screening of the same previous sera utilizing EITB revealed that all infected sera succeeded in producing clear, sharp reactions on the NC sheet. This finding revealed absolute 100% sensitivity and 100% specificity. The specificity and sensitivity of EITB in diagnosis were previously mentioned by several authors against different parasites. However, this technique is considered a time-consuming and non-applicable field test (Mahdy et al., 2017; Taher et al., 2017).

The ability to utilize a modified ELISA assay depends on using specific purified antigens that can partially overcome the drawbacks of indirect ELISA. Thus, during the present study, the assay was performed by directedly dotting the purified eluted concentrated pooled antigens from the two specific fractions at MW of 48 and 58 kDa that were previously identified by EITB on a piece of NC sheets divided by a pencil into 10 X 10 mm squares. Then, testing its diagnostic efficacy after being absorbed in the NC sheet. The assay revealed the same results as previously reported after EITB, with 100% sensitivity, specificity, and accuracy that were better than indirect ELISA. Moreover, the assay did not demonstrate false-positive reactions versus control negative human and sheep sera.

Mixing both fractions (48 and 58 kDa) as pooled antigens after elution initiated a synergistic action increasing the concentration of specific antigen per dot, causing a darker color per dot in comparison with the relatively faint reaction per band obtained per each separate specific band after EITB. After using dot-ELISA, the high protein concentration per dot enhanced the number of strong positive results while decreasing the number of weak positive reactions. With a fixed amount of protein/dot, the obtained color of the dot was directly related to the level of ACc-Ab in the tested sera, which was directly related to the mean OD values of ELISA. This result was in agreement with Taher et al. (2017).

The described dot-ELISA had all indirect ELISA benefits and overcame its drawback related to the degree of antigen purification, which minimized the level of cross-reaction with other parasites. It was more economic because it used a small number of reagents. In addition, this assay could be easily performed and could be evaluated by the naked eye or a densitometer. The test gathered the benefits of ELISA because it could be performed on a large number of samples simultaneously in a short time and had the accuracy of EITB as it used a specific purified protein fraction.

The thickness of the gel slide and the quantity of initially added antigen had an impact on the protein content of the gel strip that was produced. It just required dotting a tiny amount of protein/square of 2-3 µl/dot to run several samples using the concentrated eluted fractions that were produced (about 0.2 mg protein). Additionally, it was able to prepare any amount of pooled antigen by repeatedly fractionating and eluting the sample. The three diagnostic assays were evaluated to accurately diagnose the infection of 20 suspected patients and sheep. Statistical analysis for their diagnostic efficacy

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revealed very low PPV for diagnosis by indirect ELISA in suspected patients (28.75%), while it was 100% in suspected sheep. In the author's opinion, this finding was related to the experiences of veterinarians in diagnosing the disease from its apparent characteristic symptoms. The finding was in agreement with Desouky et al. (2011), who reported that the infection was 100% in suspected sheep. In contrast, Amer et al. (2017) revealed that the infection was 26.4% in clinically suspected sheep. Patients usually attend the clinic for several causes of headaches with numerous incriminated diseases. For this reason, the PPV in sheep was considered to be high in comparison with patients.

Both EITB and Dot-ELISA techniques excluded seven false-positive cases from the suspected patients that were previously determined as positive by ELISA. On the contrary, both methods determined three sheep as being infected more than the 12 cases previously diagnosed by ELISA. Interestingly, the four patients proved truly infected after CT and MRI sonography, and the 15 positive sheep proved that they harbored the parasite cyst in their brains at P.M. inspection post slaughtering. CT was done using a state-of-the-art 64-channel multislice machine (Toshiba Aquillion). While MRI was performed using 1.5 Tesla MR imaging unit (Intera, Philips Medical Systems, Netherlands).

In the study, the sheep were primarily used to increase the number of known positive controls and test the accuracy of the results produced by the improved dot-ELISA on symptomatic harmed living sheep and the exact results after slaughtering and examining these animals.

The small sample size was the study's principal limitation in determining the cut-off point. There was difficulty in obtaining a large number of surgically proven coenurosis-infected patients and serum samples from a large number of sheep infected by this parasite only. At the same time, the availability of reference sera from known infected patients or sheep was considered more valuable than using hyper-immune sera prepared in an experimental animal after injection by pure or crude antigens as described by Jeyathilakan et al. (2021).

CONCLUSION

Investigation of *C. cerbralies* infected or suspected human or sheep sera by EITB or dot-ELISA depending on reaction versus pooled purified 48 and 58 kDa fractions of CcS-Ag revealed absolute (100%) sensitivity and specificity. Indirect ELISA using CcS-crude antigen revealed absolute sensitivity, while its specificity reached 75% versus non-infected and other parasitic infected sera. EITB and dot-ELISA showed high accuracy in the diagnosis of infection in suspected cases proved by sonography in patients and PM inspection in sheep. The described dot-ELISA using this pooled purified antigen proved an easily applicable diagnostic tool gathering the benefits of both ELISA and EITB. The assay can be applied on the field level after producing enough of these specific fractions. Further study is continued to identify the relation between genotypes of *C. cerbralis* species extracted from humans and sheep in the near future.

DECLARATIONS

Authors' contributions

All authors consented to submit the manuscript to the current journal, gave final approval of the version to be published, and agreed to be responsible for all aspects of the work. They also significantly contributed to the conception, design, data collection, analysis, and interpretation.

Competing interests

There are no competing interests with regard to this work, according to the authors.

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Ethical consideration

The authors have checked for ethical issues, such as plagiarism, approval of public misconduct, data fabrication or falsification, duplicate publishing or submission, and redundancy.

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Impact of Colchicine on Histology of Testis in Rats

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ABSTRACT

Colchicine is a drug widely used for the management of many disorders, such as acute gout and Behçet's disease. It is also prescribed for the treatment of pericarditis, atrial fibrillation coronary artery diseases, and secondary amyloidosis. In case this drug is used at the early stages of coronavirus infection, its anti-inflammatory properties may reduce the severe inflammatory reactions related to a cytokine storm by affecting the inflammasome. The purpose of the present study was to determine the toxicity of Colchicine on testis in rats from different age groups for 10 days. A total of 27 male Wistar rats were divided into three groups. The rats in group I (control group) were administered distilled water by oral gavage. Group II consisted of young rats (5-6 months old) who orally received Colchicine 3 mg/kg body weight. Group III entailed rats of 14-16 months who were orally administered colchicine 3 mg/kg body weight. The testis of the treated groups was dissected and examined for histological changes and morphometrical analysis. The obtained results indicated that high doses of Colchicine (3 mg/kg body weight) could induce tissue damage to the testis, including degeneration and necrosis of both Sertoli and Leydig cells with irregular divisions of germinal epithelium, even when it was used for short periods (10 days). In the elderly treated rats, there were severe tissue damages, including degeneration and necrosis of germinal epithelium with irregular divisions of germ cells, necrosis of Sertoli and Leydig cells with sloughing of germinal epithelium toward the lumen of the tubule. Therefore, there is a need to conduct more studies to investigate the side effect of Colchicine as it is excessively used in the management of coronavirus.

Keywords: Colchicine, Histology, Morphometric trait, Rat, Testis

INTRODUCTION

Colchicine ($C_{22}H_{25}NO_6$) is an alkaloid extract originating from Colchicum autumnale seeds (Bertram et al., 2012). It is commonly prescribed for the management of acute attacks of gouty arthritis (Schlesinger et al., 2006), Behcet's syndrome (Yurdakul et al., 2001; Dasgeb et al., 2018), familial Mediterranean fever with associated amyloidosis (Hasbani et al., 2019). Furthermore, it is prescribed for the management of pericarditis (Schenone et al., 2018), atrial fibrillation (Siak et al., 2021), coronary artery diseases (Vaidya et al., 2019), and skin disorders (Saxena et al., 2019; Sardana et al., 2020).

It has antimitotic action by inducing the cessation of cellular mitosis in the metaphase of the cell cycle and disrupting the inflammatory pathway inducing its anti-inflammatory action (Leung et al., 2015). It is attached to tubulin for forming irreversible tubulin-colchicine complexes and induces GTPase enzymatic action to enhance the loss of the microtubule causing its depolymerization and cessation of its elongation (Bhattacharyya et al., 2008; Angelidis et al., 2018). In addition, it can arrest the meiotic divisions in murine (Liang et al., 1985). Colchicine is recently used for the treatment of covid 19 patients (Lopes et al., 2021; Scarsi et al., 2020) due to its anti-inflammatory action (Li et al., 2021; Kurek et al., 2021). It causes alterations in many inflammatory reactions as it prevents the aggregations of macrophages at the site of inflammation-reducing cytokine release and production of superoxide by neutrophils (Martínez et al., 2018; Kamel et al., 2021; Vitiello et al., 2022).

Oral Colchicine is quickly absorbed by the gastrointestinal system, and then, is metabolized and processed in the hepatic and intestinal system through cytochrome (P450), P-glycoprotein, and demethylated to major metabolites named demethylcolchicine (Mizutani et al., 2008). It is excreted primarily through the intestinal and biliary systems. Only about 10-20% were eliminated by the renal system (Niel and Scherrmann, 2006). The current study aimed to determine the damaging effect of Colchicine on the testis by comparing the young treated rats with elderly ones.

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MATERIALS AND METHODS

Ethical Committee Approval

Ethical approval was sought and accepted from the medical research ethical committee of Mosul University, Mosul, Iraq (UOM/COM/MREC/21-22(11).

Drug

The drug was obtained from Cipla Medpro, India, with the brand name Colchicine (1 mg).

Experimental animals

A total of 27 male Wistar rats with a weight mean of 200 g were purchased from the experimental animal section in the Veterinary College/University of Mosul, Iraq. The rats were randomly divided into three groups with three replicates for each group. Group I was assigned as the control group, group II included young rats within the age range of 5-6 months, and group III entailed rats aged 14-16 months. Animals were housed in plastic cages (three per cage) in standard humidity and at room temperature maintained at 25°C. A controlled 12-hour light-dark cycle was maintained and they were given the diet *ad libitum* and normal tape water. Animals fed a balanced diet which was produced by the research council of the Veterinary College of Mosul University, Iraq.

Dosage treatment

Group I (control group) received distilled water orally while groups II and III were administered Colchicine 3 mg/kg body weight daily for 10 days which was double therapeutic dose. All groups received the medicine by oral plastic gavage (15g/78 mm).

Histology

The treated animals were sacrificed by diethyl ether 2 days after the last dose of Colchicine (day 12 of the experiment) was administered. The testis was excised and fixed with 10% neutral buffered formalin. Specimens were dehydrated by alcohol, cleared by clearing agent xylene, and blocks of paraffin wax were made. Then, paraffin sections were obtained and cut into 5-micrometer thickness. Finally, sections were stained with hematoxylin and eosin and examined under a light microscope (Olympus, Germany) in Al-Mahmood Laboratory for Histopathological Consulting, Mosul, Iraq.

Histomorphometric approaches

The investigated parameters in the current study included the diameter of the seminiferous tubule (µm), thickness of the germinal layer (µm), and perimeter of the seminiferous tubule (µm). The number of Sertoli cell/tubule and number of Leydig cell/tubule were measured using 40x magnification of the microscope. A specialized digital camera (OMAX 18 MP, China) with USB 3.0 was used for morphometric estimation.

Statistical analysis

The morphometrical study was estimated by Sigma plot V12.0 / SYSTAT software. Parameters were determined as mean with standard error and evaluated by ANOVA test with significance at $p < 0.05$. Duncan's test was used to assess differences among the mean of groups (Kirkwood, 1988).

RESULTS

The tissue sections of the testis of group I showed normal histological features, including organized shape and size of seminiferous tubules, regular division, and maturation of spermatogonia cells. Normal germ cell proliferation and mature sperms were shown in the lumen of the tubules (Figure 1). The microscopic examination of histological sections of the testis of group II showed degeneration and necrosis of Sertoli cells (Figure 2). The findings indicated the irregular division of germinal epithelium with vacuolar degeneration of spermatid cells (Figure 3). There was interstitial edema between the seminiferous tubules with degeneration and necrosis of Leydig cells (Figure 4). As can be seen in Figure 5, there were vacuolar multinucleated giant cells and seminiferous tubules were free from sperms containing only cellular debris. The microscopic examination of tissue testis of group III showed more severe histopathological changes than group I, as it was characterized by degeneration and necrosis of germinal epithelium with severe vacuolation of spermatogonia cells (Figure 6). Irregular division of germ cells lining the tubules with necrosis of Sertoli cells and deformed, abnormal spermatocytes and spermatids were noticed. The lumen of seminiferous tubules was obstructed by cellular debris (Figure 7). Degeneration and necrosis of Leydig cells with interstitial edema and dilatation of seminiferous tubule lumen occurred due to the degenerative process (Figure 8). There was damage to the basement membrane of the tubules with sloughing of germinal epithelium toward the lumen of the tubule (Figure 9).

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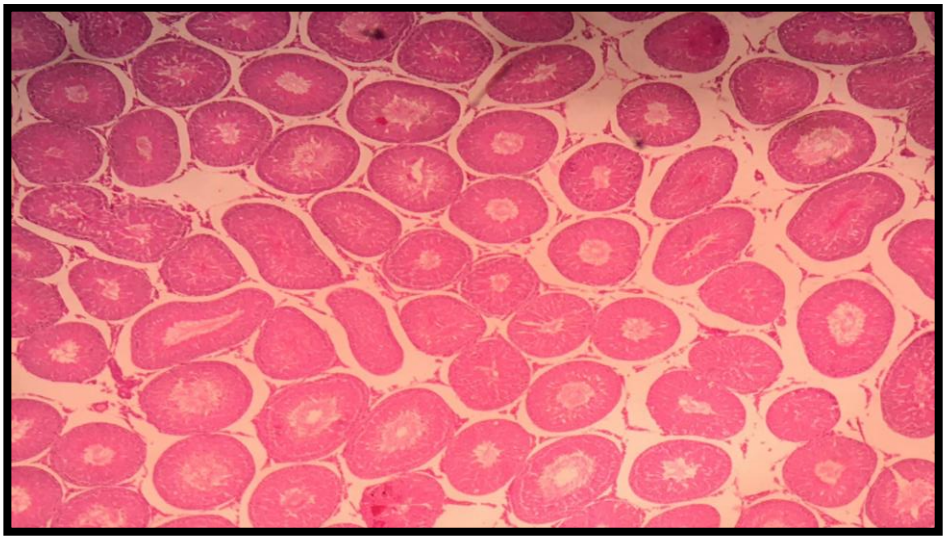

Figure 1. Normal architecture of testis in young rats (5-6 months old) with normal looking seminiferous tubules (H&E, 100X)

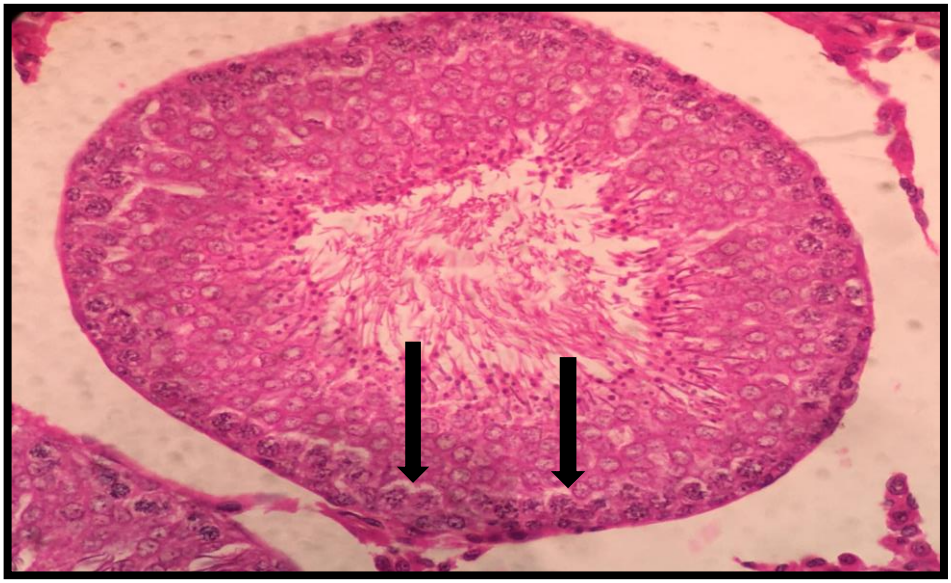

Figure 2. Degeneration and necrosis of sertoli cells in the young age treated rats (5-6 months old) with Colchicine (Black arrows, H&E stain, 400X)

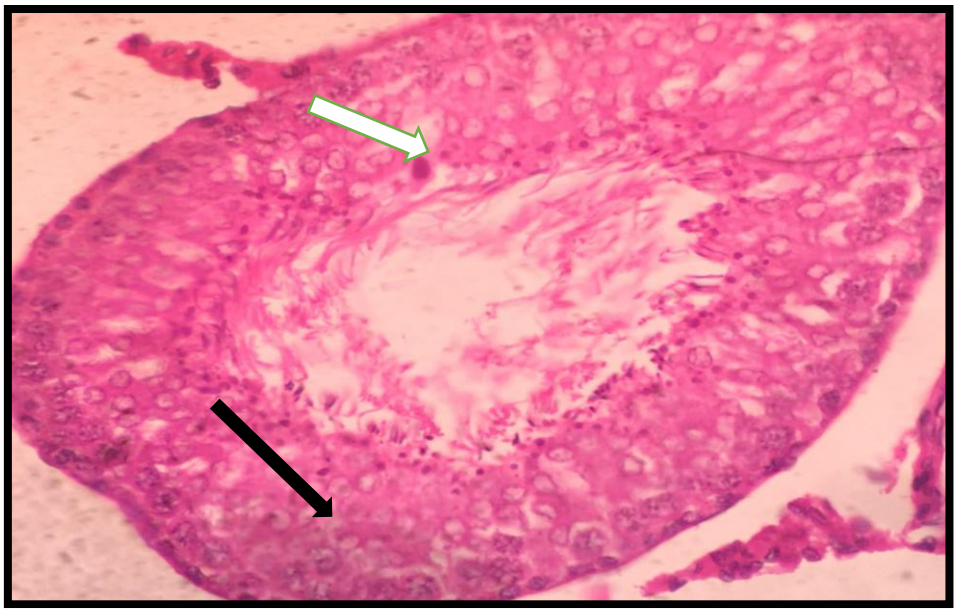

Figure 3. Irregular divisions of germinal epithelium (black arrow) in a young age rat (5-6 months old) treated with Colchicine. Vacuolar degeneration of spermatid cells (White arrow, H&E stain, 400X)

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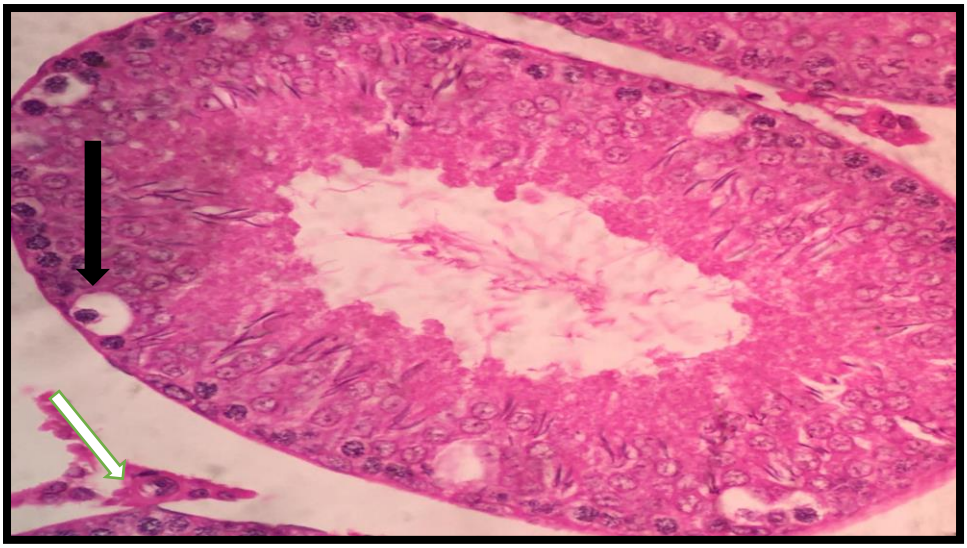

Figure 4. Vacuolar degeneration of spermatid cells (black arrow) in a young age rat (5-6 months old) treated with Colchicine. Degeneration of Leydig cells (White arrow, H&E stain, 400X)

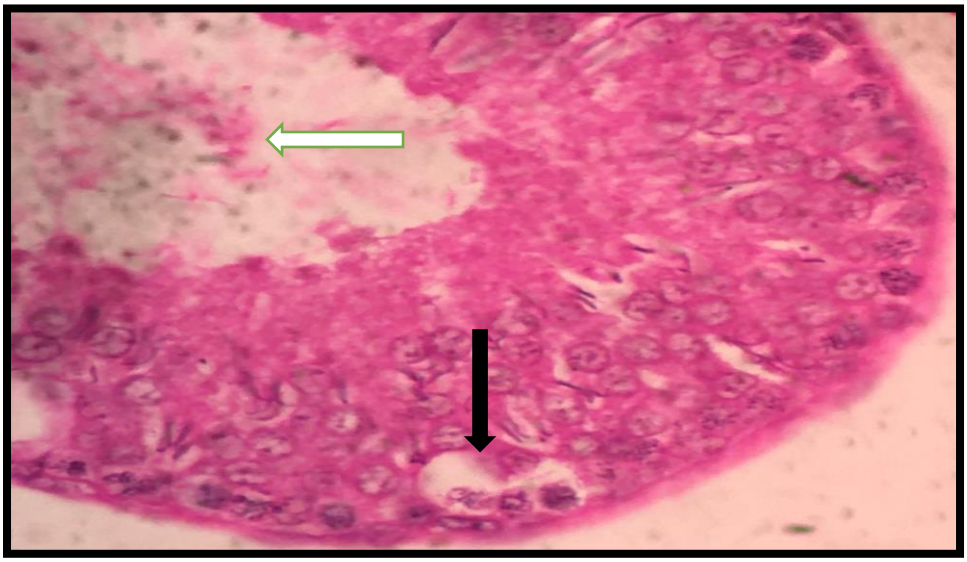

Figure 5. Vacuolar multinucleated giant cells (black arrow) in a young age rat (5-6 months old) treated with Colchicine. Cellular debris in the lumen without sperms (White arrow, H&E stain, 400X).

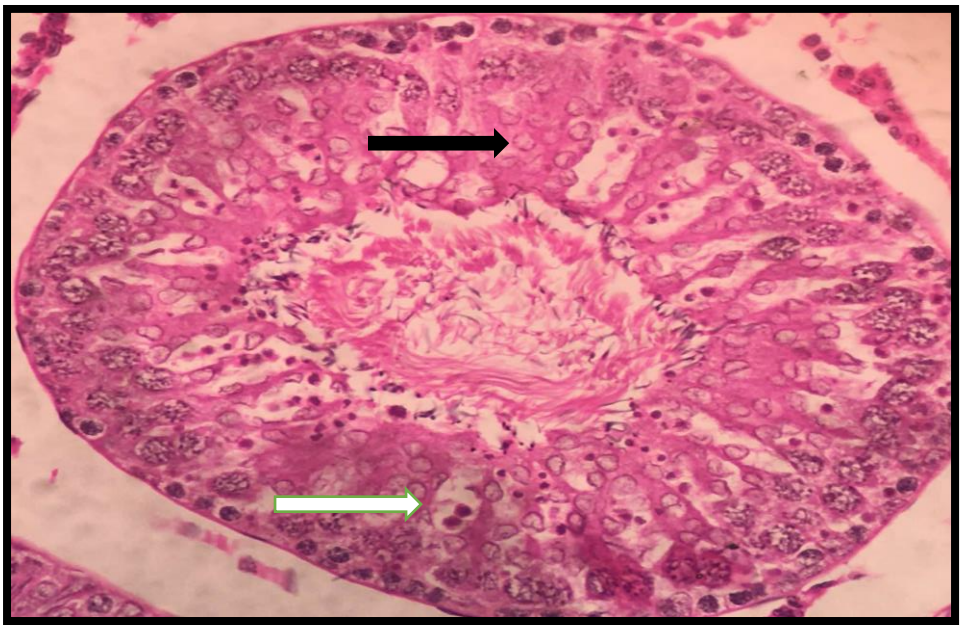

Figure 6. Degeneration and necrosis of germinal epithelium (black arrow) in old rats (14-16months old) treated with Colchicine. Severe vacuolation of spermatogonia cells (White arrow, H&E stain, 400X).

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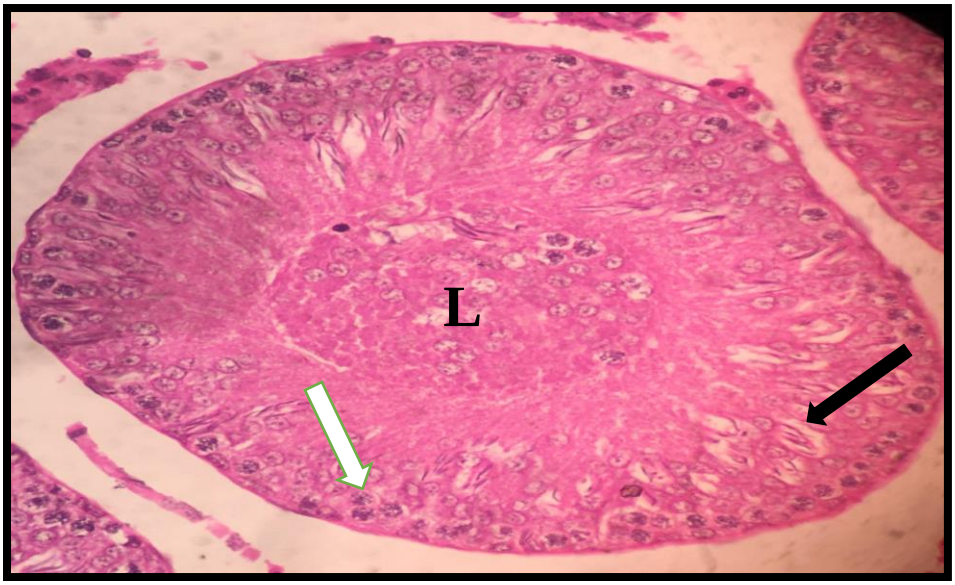

Figure 7. Irregular divisions and abnormal shape of germ cells (black arrow) with necrosis of Sertoli cells (white arrow) in an old rat (14-16 months old) treated with Colchicine. Obstructed lumen by cellular debris (L, H&E stain, 400X).

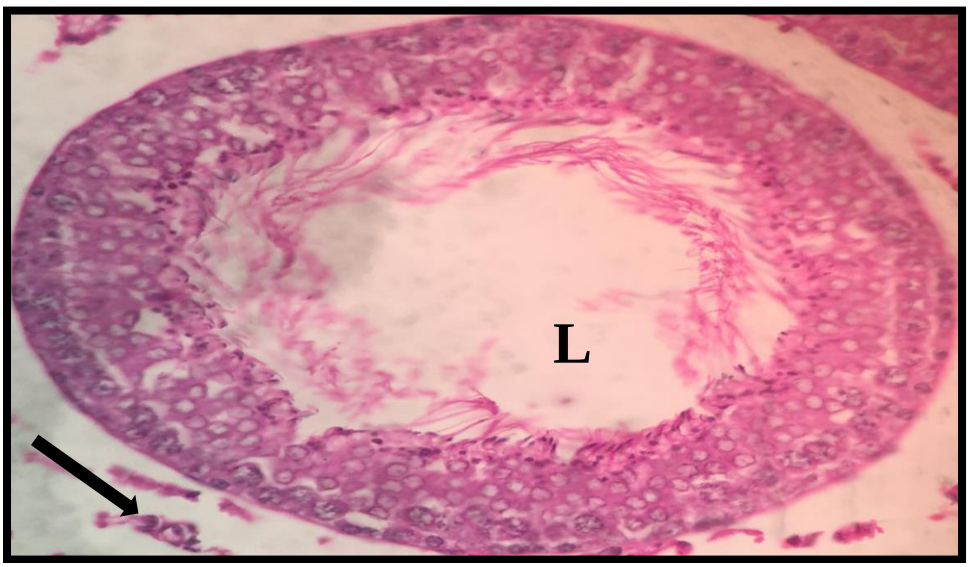

Figure 8. Degeneration and necrosis of Leydig cells (black arrow) in an old rat (14-16 months old) treated with Colchicine. Dilated lumen with debris (L, H&E stain, 400X)

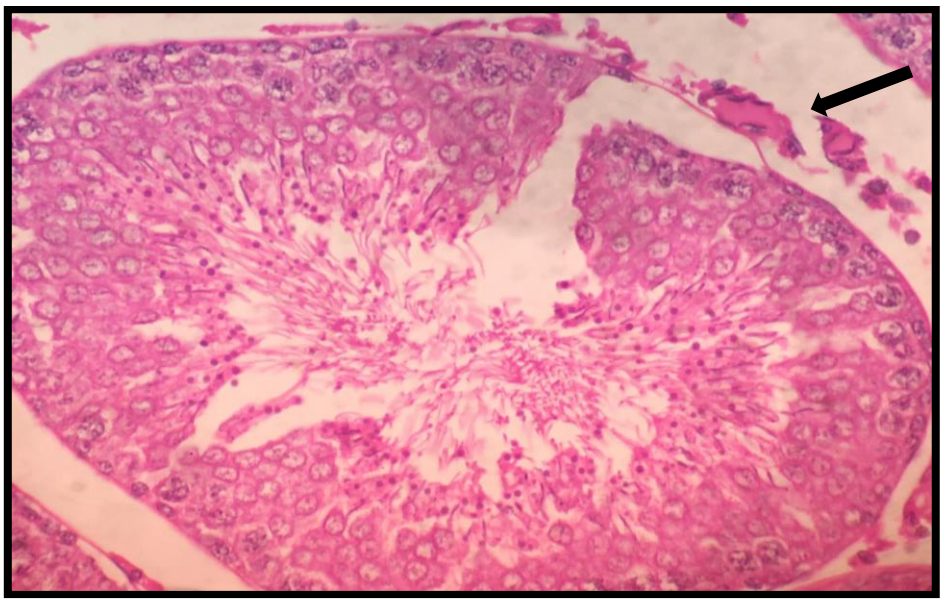

Figure 9. Damage of the basement membrane of the seminiferous tubules in an old rat (14-16 months old) treated with Colchicine (black arrow, H&E, 400X)

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Morphometric analysis

Seminiferous tubules diameter

The diameter of the seminiferous tubules/ µm of group I (control group) was 247.9 ± 4.7. The diameter of young age-treated rats (group II) was 224.92 ± 5.3. However, in the old age-treated rats (group III), the diameter was 184.84 ± 4.2 (Table1, Graph 1).

Germinal epithelium thickness

The germinal epithelium thickness / µm of group I (control group) was (80.94 ± 2.5). The thickness was 78.234 ± 1.9 in group II, it was 62.34 ± 1.6 in group III (Table 1 and Graph 1).

Seminiferous tubules perimeter

The seminiferous tubules perimeter/µm was reported as 40347.56 ± 986.4, 39901.42 ± 734.9, and 34718.3 ± 917.3 in groups I, II, and III, respectively (Table 2 and Graph 2).

Sertoli cell

The number of Sertoli cell/tubules in groups I, II, and III was measured as 11.2 ± 0.8, 11.6 ± 0.5, and 9.8 ± 0.3, respectively (Table 3 and Graph 3).

Leydig cell

Regarding the number of Leydig cells/40x field, group I indicated 10.2 ± 0.5, while this number was 8.2 ± 0.7 and 5.2 ± 0.3 in groups II and III, respectively (Table 3 and Graph 3).

Table 1. Diameter of seminiferous tubule and the thickness of the germinal epithelial layer in rats with different age ranges treated with Colchicine

Groups \ Treatment	Control	T1	T2
Seminiferous tubules diameter (µm)	247.9 ± 4.7[a]	224.92 ± 5.3[a]	184.84 ± 4.2[b]
The germinal epithelium thickness (µm)	80.94 ± 2.5[a]	78.234 ± 1.9[a]	62.34 ± 1.6[b]

Different superscript letters in the same row explain a significant statistical difference at $p < 0.05$. T1: Young rats (5-6 months old) were administered Colchicine 3 mg/kg body weight, T2: Old rats (14-16 months old) were administered Colchicine 3 mg/kg body weight

Table 2. Seminiferous tubule perimeter in rats with different age ranges treated with Colchicine

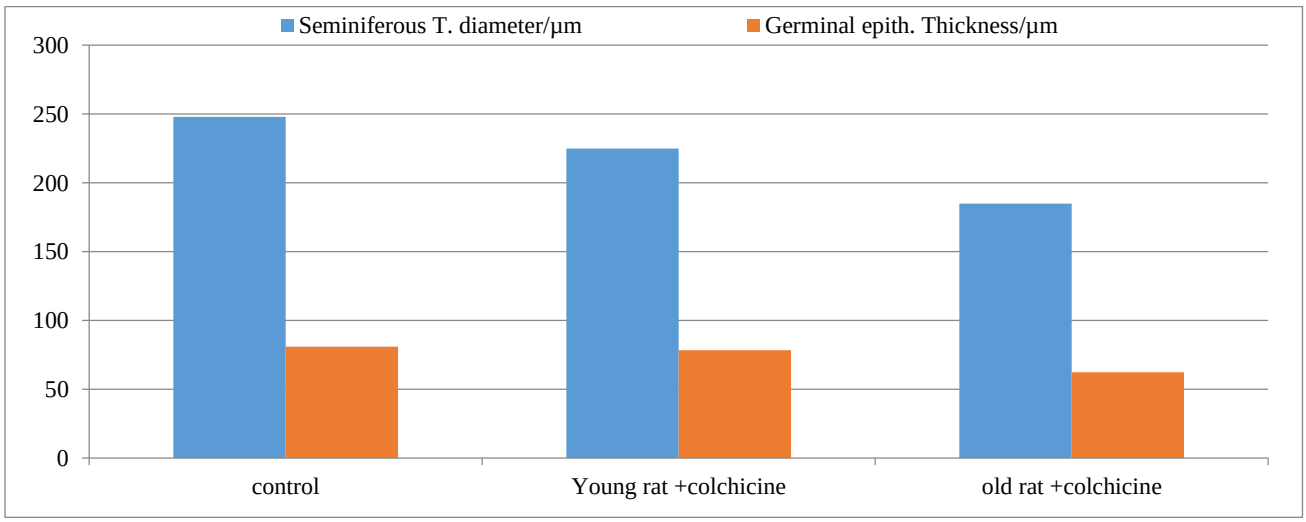
Groups \ Treatment	Control	T1	T2
Seminiferous tubules perimeter/µm	40347.56 ± 986.4[a]	39901.42 ± 734.9[ab]	34718.3 ± 917.3[b]

Different superscript letters in the same row explain a significant statistical difference at $p < 0.05$. T1: Young rats (5-6 months old) were administered Colchicine 3 mg/kg body weight, T2: Old rats (14-16 months old) were administered Colchicine 3 mg/kg body weight

Table 3. Sertoli cell and Leydig cell in rats with different age ranges treated with Colchicine

Groups \ Treatment	Control	T1	T2
Sertoli cell No./tubule	11.2 ± 0.8 [a]	11.6 ± 0.5 [a]	9.8 ± 0.3 [b]
Leydig cell No./40x field	10.2 ± 0.5 [a]	8.2 ± 0.7 [a]	5.2 ± 0.3 [b]

Different superscript letters in the same row explain a significant statistical difference at $p < 0.05$. T1: Young rats (5-6 months old) were administered Colchicine 3 mg/kg body weight, T2: Old rats (14-16 months old) were administered Colchicine 3 mg/kg body weight



Graph 1. Diameter of seminiferous tubule and the thickness of the germinal epithelial layer in rats with different age ranges treated with Colchicine

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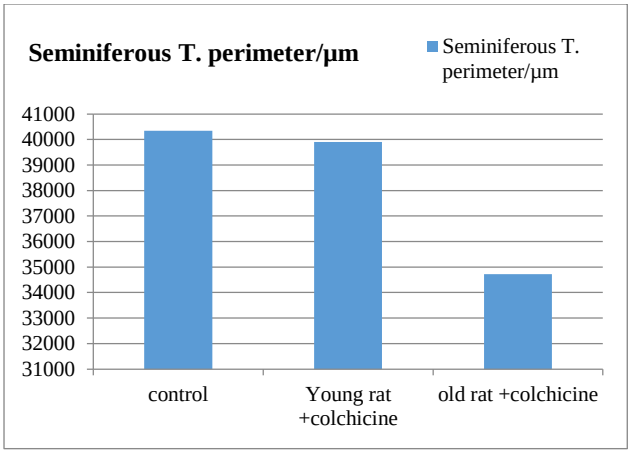
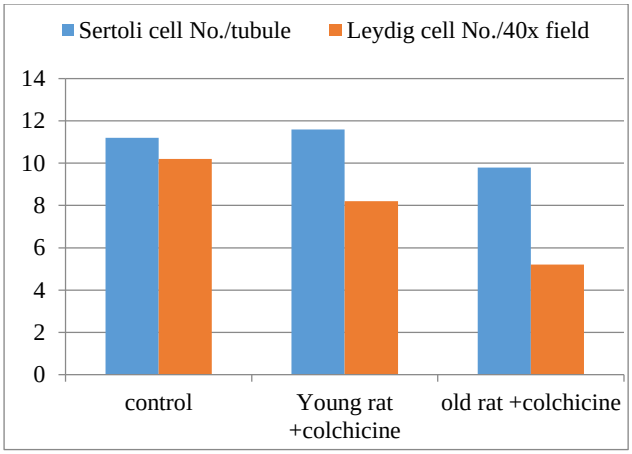


Graph 2. Perimeter of Seminiferous tubules in rats with different age ranges treated with Colchicine



Graph 3. Sertoli cell and Leydig cell in rats with different age ranges treated with Colchicine

DISCUSSION

Colchicine has a narrow therapeutic index, meaning that it is a safe drug as long as it is used within the therapeutic dose but toxic in higher doses (Maxwell et al., 2001). It is widely used in the management of many disorders, such as like gout, but nowadays, it is used in the treatment of coronavirus patients as it has an anti-inflammatory action that reduces respiratory damage (Gómez et al., 2021).

Considering the obtained results of the current study, young rats in Group II showed some pathological changes, such as degeneration and necrosis of Sertoli cells, and irregular divisions of germinal epithelium vacuolar multinucleated giant cells. The lumen of the seminiferous tubules was free from sperms.

Unlike other groups, group III (the old age-treated rats) demonstrated more severe pathological changes, including degeneration and necrosis of the germinal epithelium. Moreover, there were irregular divisions with the abnormal shape of spermatocytes and spermatids with necrosis of Sertoli and Leydig cells. The lumen of seminiferous tubules was obstructed by cellular debris. Damage to the basement membrane of the tubules with sloughing of the germinal epithelium was also detected.

Concerning Morphometric analysis, all the parameters of group III showed a significant decrease. However, young age treated rats (group II) indicated a non-significant difference when compared to group I. These changes were inconsistent with Abdel Motaal's (2006) results who found that Colchicine used (3 mg/kg body weight) for a few days can cause degeneration and necrosis of the sperm cells, Sertoli cells and Leydig cells with associated interstitial edema. In addition, debris was found within the lumen.

These results were in line with the findings of a study by Handel (1979), indicating the intratesticular administration of Colchicine for a short period (5 days) induced the degradation of microtubules. Consequently, the abnormal shape of the sperm which was associated with abnormal function.

In the present study, there was a cessation of the proliferation process of spermatocytes, and some sections showed lumen filled with debris and free from sperms. The findings were in agreement with previous studies (Kochman, 1998; Ben-Chitret et al., 1993)**,** indicating that Colchicine can lead to oligospermia and even azoospermia through its effect on microtubules.

The results of the current study coincided with the results of a study by Abbas et al. (2016), who studied the effect of high dose (25mg/kg) Colchicine treatment in rats, and they found that Colchicine induced some pathological changes, including degeneration of germinal epithelium, and sloughing of the basement membrane of the tubules with an accumulation of the debris in the lumen as well as the cessation of the process of spermatogenesis.

These findings were in agreement with those of Shehab et al. (2014) and Elshama et al. (2013) results who recorded that Colchicine could enhance the degeneration of seminiferous tubules as well as Sertoli and Leydig cells with vacuolar degeneration of sperm cells with abnormal shape and cessation of proliferation process leading to azoospermia.

Similar to the results of the present study, Russel et al. (1981) stated that Colchicine could induce seminiferous tubules damage, sloughing of sperm cells within the lumen with cessation of sperm proliferation process, and degeneration of Sertoli cells in the testis of rats. In addition, they stated that the destruction of Sertoli cells was responsible for sloughing the neighboring germ cells into the lumen. Allard et al. (1993) studied the dose-related effect of Colchicine on rat testis and found that intratesticular administration of higher doses (40 µg) of Colchicine can lead to damage to the seminiferous tubules.

Colchicine has anti-inflammatory action as it reduces the production of free radicals and oxidative stress but it produces its effect by decreasing the polymerizing process of the microtubules leading to its destruction (Prins et al.,

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2017; Golpour et al., 2021). A study by Sarica et al. (1995) indicated that Colchicine could affect spermatogenesis and lead to the cessation of the proliferation process and azoospermia.

The results of the present study were consistent with those reported by Correa et al. (2002). They studied the way Colchicine and carbendazim could affect testicular tissue in mice and they found that low dose intratesticular injected mice were normal while high dose treated mice showed damage in the seminiferous tubules. The associated morphometric study showed a significant statistical decline in the number of tubules due to sloughing the germinal epithelial layer.

CONCLUSION

The present study concluded that high doses (3 mg/kg body weight) of Colchicine could produce tissue damage to the testis even when it is used for a short period (10 days), and the tissue damage was more severe in the elderly treated rats, compared to younger ones.

DECLARATIONS

Acknowledgments

We are glad to acknowledge animal house manager Dr. Ahmed Nadhem Hassan for his support.

Authors' contribution

Rand Abdulateef Abdullah carried out the idea of the article, calculated the doses, handled the experimental animals, administered the medicine, and wrote down the whole article. Hana Khaleel Ismail was responsible for (results chapter), examining the tissue sections and identification of pathological changes in all groups. Ammar Ghanim Al-Haaik carried out a morphometrical study, assessment of all parameters using a specialized camera, and analyzing morphometrical data, and graphs. All authors checked and approved the final version of the manuscript.

Competing interests

No conflict of interest has been declared by the authors.

Ethical considerations

Plagiarism, consent to publish, misconduct, data fabrication and or falsification, double publication and or submission, and redundancy have been checked by the authors.

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Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on *Vibrio Parahaemolyticus* in Shrimps

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ABSTRACT

Recently, there has been a tremendous increase in the studies addressing the application of bioactive compounds from the natural ecosystem, particularly for medical purposes. Hence, the present study investigated the antibacterial properties of the secondary metabolites possibly contained in the leaves of *Avicennia marina* (*A. marina*) for possible prevention of *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a devastating bacterial pathogen in shrimp aquaculture. In the current study, secondary metabolites were extracted from the leaves of mangrove plant using ethanol extraction method. The ethanolic extracts were then subjected to phytochemical and antibacterial activity tests. The results from the phytochemical analysis demonstrated that the ethanolic extract from the mangrove plant contained varying amounts of flavonoids, tannins, saponins, polyphenols, alkaloids, steroids, and triterpenoids. However, the number of flavonoids and alkaloids seemed to be higher than the other metabolites. The antibacterial activity analysis through the agar diffusion method has shown that different concentrations (50 ppm, 100 ppm, 200 ppm, and 300 ppm) of the ethanolic extract of *A. marina* inhibited the *V. parahaemolyticus.* At 300 ppm, the plant extract exhibited 17.3% antibacterial effectiveness, compared to the antibacterial activity of chloramphenicol. The findings indicated that the secondary metabolites of *A. marina* have the potential that can be developed as an alternative treatment for aquatic animal diseases in the future.

Keywords: Aquaculture, Bioactive compounds, Mangrove ecosystem, Treatment

INTRODUCTION

The application of medicinal plants as phytopharmica products in place of synthetic antibiotics has been increasingly popular in recent years. Many of these products are sold over the counter or online retailing. However, many plant species in tropical countries, including Indonesia, are still not thoroughly investigated for their medicinal properties due to their high diversity. Some of these plants are known to have bioactivity against disease-causing pathogens. For example, the tiwai onion (*Eleutherine Americana*) has been shown to strongly inhibit the growth of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) and *Vibrio harvey* (Azis, 2019; Azis and Cahyadi, 2020).

Similarly, the Dragon Scales Leaf (*Drymoglossum pilosellaoides*) was also reported to contain antibacterial properties against *V. parahaemolyticus* (Azis, 2019). This antibacterial activity is influenced by the presence of various bioactive compounds in phytopharmaceutical extracts that can damage bacterial cell walls causing a modification of the cytoplasmic membrane, the release of core material, changes in protein, and nucleic acid molecules, enzyme inactivity, and preventing protein and nucleic acid synthesis. Interactions include sequential inhibition following common biochemical pathways to enhance antimicrobial diffusion, and inhibition of bacterial protective enzymes (Sintayehu et. al., 2022). Based on toxicity study, antibacterial substances can be classified in three forms, namely bacteriostatic, bactericidal, and bacteriolytic (Parekh and Chanda, 2007). The progress of the bioactivity study of plants is concentrated on plants primarily found in terrestrial ecosystems; however, few studies have been conducted on the plants that are flourishing in the marine ecosystem. Mangrove plants, including *Avicennia marina*, are rich in bioactive substances and have potential as an agent in various biological activities (Al-Mur, 2021). Many of these marine plant species are known

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to contain unique bioactive compounds in the leaf extract of a mangrove plant, including *Avicennia alba* leaf extract can prevent the development of cancer cells (Eswariah et. al., 2020).

A total of 81 mangrove species have been reported worldwide, and several biologically active compounds have been isolated from mangroves with various levels of action that have biomedical potential, such as anticancer, antiulcer, antioxidant, antidiabetic, and antimicrobial (Parthiban et al., 2021). One of the most common and easily found mangrove species in Indonesia is the *Avicennia marina*. This species has been extensively used by the indigenous peoples for many purposes, including materials for building the house and traditional medicine for skin diseases, rheumatism, ulcers, and smallpox (Bandaranayak, 2002). Due to its extensive use in traditional medicines, the extracts of this plant are assumed to contain diverse bioactive compounds that could benefit the pharmaceutical industry (Nurjannah et al., 2015).

This *Avicennia* species is predominantly distributed in Tarakan, North Kalimantan (Mahera et. al., 2011). Its leaves, skin, and fruits are highly valuable because of their medicinal properties (Huang et. al., 2016; Oktavianus, 2013). In addition, the bark of the plant can also be further processed to generate many more downstream products, such as alcohol, hydrocarbons, carbohydrates, inorganic salts, minerals, phytoalexins, vitamins, iridoid glucosides, steroids, tannins, triterpenoids, as well as fatty, amino, and carboxylic acids (Ananthavalli and Karpagam, 2017). Biologically active compounds, such as limonoids, terpenoids, alkaloids, glycosides, steroids, flavonoids, esters, quinones, phenols, acids, aliphatic alcohols, amides, lactones, aliphatic ketones, and benzodioxols have been extracted from leaves, stems, bark, fruits, and seeds of Indian mangroves (Parthiban et al., 2021). Overall, this study confirms the antimicrobial and antioxidant activity of mangrove ethyl acetate extract (MEE) and inhibitory and eradicating activity of the *A. marina* biofilm ethyl acetate extract against *Pseudomonas fluorescens* (Ibrahim et al., 2022).

Furthermore, several in-vitro studies have shown the leaf extract of *Avicennia* is highly potent against various pathogenic bacteria *Virgibacillus marismortui* and *Micrococcus iuteus* (Ulmursida et al., 2017). However, most previous studies on the medicinal properties of *A. marina* focused only on human illnesses. A few addressed the use of this plant towards diseases of farmed aquatic animals, such as fish and shrimps. One of the major disease-causing bacteria in shrimps is the V. parahaemolyticus. This halophilic Gram-negative bacterium is ambiguous in the marine environment. This bacterium has been known to cause disease in shrimp aquaculture in North Kalimantan. Mangroves are coastal plants that can adapt and survive in intertidal tropical and subtropical coastal areas, where their presence in marine ecosystems is the second most important after coral reefs (Al-mur, 2021). The bacterium causes acute damage to the digestive system of culture shrimp, particularly the hepatopancreas. *Vibrio parahaemolytics* have also been widely reported to trigger early mortality syndrome (EMS) or the acute hepatopancreatic necrotic disease (APHND) in culture shrimp worldwide. Furthermore, it has also been reported to be responsible for vibriosis in marine crabs (Jithendran et al., 2010).

The widespread use of *A. marina* for traditional medicine might also be helpful for aquatic animal husbandry, particularly for preventing vibriosis in cultured shrimp. Therefore, the present study aimed to examine the antibacterial potential of the ethanolic leaf extract of the *A. marina* towards *V. parahaemolyticus*, a devastating pathogen of culture shrimp.

MATERIALS AND METHODS

The extraction process, bacterial culture, antibacterial activity test, and observation of bacterial growth inhibition zones were all carried out at the Laboratory of Nutrition and Fish Feed, Faculty of Fisheries and Marine Sciences, University of Borneo Tarakan. The leaves of *A. marina* were obtained from the Mangrove and Proboscis Monkey Conservation Area (KKMB) of Tarakan City (Figure 1). Meanwhile, the isolates of *Vibrio parahaemolyticus* were acquired from the Center for Brackish Water Aquaculture Fisheries (BBPBAP) Jepara, Indonesia, with the number of bacterial isolates VpPm 110321-3.

Figure 1. The bioactive compounds of *A. marina* leaf samples were extracted according to Manila et al. (2009) with the modification and antimicrobial activity of extracts of *A. marina* examined against the pathogen.

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Sample preparation and extraction

Crude extract of the leaves of *A. marina* was prepared following the method suggested by Manila et al. (2009). Briefly, the leaves of *A. marina* were cut into small pieces and air dried for 5-7 days. After that, the dried leaves were then finely blended and sifted. The crude preparation began by weighing 200g powder of the finely blended dried leaves of A. marina, macerated three times each for 24 hours with 200 ml of 70% ethanol solution. The solution was then stirred for 1 hour at 45°C using a hot plate with a stirrer. Subsequently, the extracts were filtered using filter paper and concentrated with a rotary evaporator for 2 hours at 40°C.

Finally, the extract solutions were dissolved in 70% ethanol solvent and diluted into different concentrations, and the groups were named A (50 ppm), B (100 ppm), C (200 ppm), and D (300 ppm). Two control solutions were also prepared alongside the plant extract, namely 70% ethanol (C-), and 0.01% chloramphenicol (C+). Each concentration of the solution was prepared in triplicates. The chloramphenicol solution was used as a benchmark or reference (positive control) to determine the effectiveness of the antibacterial activity of the ethanolic leaf extract of *A. marina*. In addition to chloramphenicol, other antibiotics used as positive controls were ampicillin (Amp), ciprofloxacin (Cip), nitrofurantoin (Nit), gentamicin (Gen), oxytetracycline (Otc), tetracycline (Tet), and streptomycin (Str).

Phytochemical tests

The phytochemical assay of *A. marina* leaf extract is briefly described below

Flavonoid Test (Harborne 1998)

To conduct this test, 1 milliliter of *A. marina* leaf extract solution was put into a test tube, and then a little magnesium powder and a few drops of concentrated HCL (Shinoda reagent) were added. If the color of the solution changes to orange, pink, or red, it indicates the presence of flavonoid compounds in the sample.

Saponin Test (Harborne 1998)

Two milliliters of *A. marina* leaf extract solution were put into a test tube and then homogenized for several minutes. The formation of persistent and stable foam for 15 minutes indicates that the sample contains saponins.

Polyphenol (Tannin) Test (Zohra et al., 2012)

One milliliter of *A. marina* leaf extract solution was put into a test tube, followed by the addition of a few drops of 5% ferric chloride ($FeCl_3$) reagent. If a brown precipitate forms, this indicates that the sample contains tannins.

Alkaloid Test (Harborne 1998)

Avicennia marina leaf extract as much as 1 milliliter was put into a test tube, and then 2-3 drops of Dragendorph reagent were added consisting of (Nitrooxy) oxobismuthine (BiNO4xH2O), tartaric acid, and KI. The formation of an orange precipitate indicates the presence of alkaloids in the sample.

Steroids and Triterpenoids Test (Zohra et al., 2012)

One milliliter of *A. marina* leaf extract solution was put into a test tube, followed by the addition of 3-5 drops of chloroform, 3-5 drops of acetic anhydride, and 10 drops of concentrated sulfuric acid. The presence of steroids is seen in the change in the color of the sample from blue to green. The presence of triterpenoids in other parts is seen by changing the color of the sample from brown to reddish brown.

Bacterial culture and antibacterial activity

Culture of *V. parahaemolyticus* was carried out on Thiosulfate citrate bile salts sucrose (TCBS) agar media. Inoculation of the bacteria was done by taking a loopful of bacterial culture using a sterile metal loop and then smeared it onto the surface of the media agar. The inoculated agar media were then incubated for 24 hours at 37°C. Antibacterial activity of the leaf extract of the *A. marina* was examined using the disc diffusion method. Briefly, a loopful of the test bacteria culture was streaked evenly on an agar medium and labelled accordingly. Then, sterile paper discs (zone diameter were soaked in the different concentrations of the plant extract solution, negative control (C-) and positive control (C+), and subsequently incubated for 24 hours at 37°C.

Observation of bacterial inhibition zone

The antibacterial activity of the *A. marina* leaf extract was ascertained by the presence of a clearing zone around the paper discs in each treatment. The diameter of the inhibition zone reflected the strength of the antibacterial activity of the plant extract. The average inhibition zone was obtained through vertical and horizontal measurements of the clearing zones.

Efficacy of antibacterial activity

The effectiveness of the antibacterial activity of the leaf extract against the test bacteria was calculated following the formula suggested by Ghosh et al. (1997). The value of antibacterial effectiveness was obtained from the division of the average diameter of inhibition zone due to plant extract (mm) by the average diameter of inhibition zone due to antibiotic (mm) multiplied by one hundred.

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RESULTS AND DISCUSSION

Based on the results of testing the inhibitory activity of *A. marina* ethanol and chloroform extracts from the Mangrove and Proboscis Monkey Conservation Area (KKMB) of Tarakan City locations, it was shown that the extract had antibacterial potential against *Vibrio parahaemolityus* bacteria. Potency is indicated by the size of the clear zone around the paper disc (Figure 2). Based on the phytochemical tests of the leaf extract, it was evident that the leaves of *A. marina* contained flavonoids, phenols, alkaloids, saponins, tannins, and steroids (Table 1). The results for the antibacterial activity of the ethanolic leaf extract of *A. marina* are presented in Table 2.

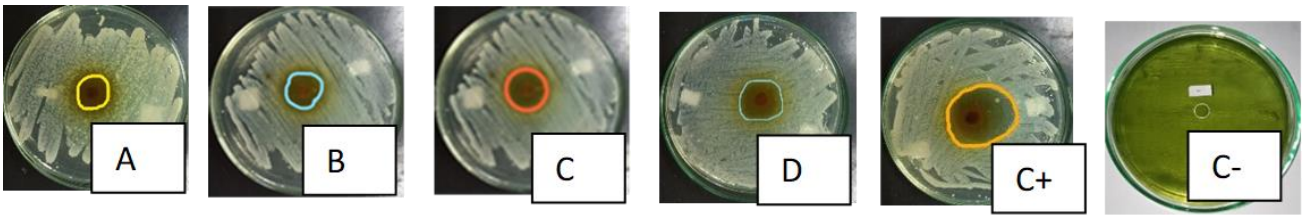

Figure 2. Inhibition zone of the crude extract of *Avicennia marina* against *Vibrio parahaemolyticus.* Treatment of *Avicennia marina* extracts*;* A (50 ppm), B (100 ppm), C (200 ppm), and D (300 ppm). Two control solutions were ethanol 70% (C-), and chloramphenicol (C+) 0.01%.

Table 1. Phytochemical test results of the crude ethanol leaf extract of *Aveciana marina* based on the strength category

Group of compound	Ethanol extracts
Flavonoid	++
Triterpenoids	+
Alkaloids	++
Saponins	+
Tannins	+
Steroids	+

+: Weak; ++: Strong; +++: Very strong based on published data of Marlinda et al. (2012)

Table 2. Inhibition zones of the different concentrations of the crude ethanol leaf extract of *Avicennia marina* against *Vibrio parahaemolyticus*

Treatment	Inhibition zone (mm)			Average (mm)	Inhibitory effectiveness (%)	Growth inhibition response
	1	2	3			
C+	35.5	37.8	37.5	36.9	100	Very strong
C-	0	0	0	0	0	No activity
A	3.4	3.5	3.4	3 .4	9.2	Moderate
B	2.8	3.9	3.7	3.5	9.5	Moderate
C	4.7	4.4	3.8	4.3	11.7	Strong
D	8.3	5.6	5.4	6.4	17.3	Strong

C+: 0.01% Chloramphenicol C-: 70% ethanol, A: 50 ppm, B: 100 ppm, C: 200 ppm, D: 300 ppm. Very strong > 80-100%, Strong > 50-79.9%, Moderate > 10-49.9%, Weak 1-9.9%, No activity 0-0.9% (Marlinda et al., 2012)

The results of this study showed that the treatment of *A. marina* leaf extract 200 and 300 ppm showed strong inhibition of *V. parahaemolyticus*. According to the results of the current study, the content of flavonoids and alkaloids seems to be abundant in *A. marina* leaves. As Edu et al. (2017) mentioned, many alkaloids are found in the roots, leaves, and bark of the *Avicennia* species. They also noted that saponins and tannins were found less in the leaves, which was the case in this study.

The organic leaf extract of *Avicennia* species has inhibitory activity against *V. parahaemolyticus* (Edu et al., 2015; Sachithanandam et al., 2019; Okla et al., 2019). Besides being rich in secondary metabolites, mangrove leaves are also known to contain minerals, vitamins, and amino acids that are important for the nutrition of marine organisms in the mangrove ecosystem (Bandarayake, 2002). Many mangrove plants have been recently used in traditional medicine Extracts of mangrove plants have antibacterial activity against human, animal, and plant pathogens. In addition, the secondary metabolite components of mangrove plants were shown to be positively correlated with the potential of the extract to inhibit bacterial growth. Phytochemical compounds such as flavonoids, phenols, alkaloids, saponins, tannins, and steroids exhibit superior antimicrobial activity against disease-causing pathogens (Sulastrianah et al., 2014). Although these chemical compounds are present in every plant, their distribution may vary according to the species and part of the plant. According to Ningsih et al. (2013), the leaf extract of *Avicennia* species has also been shown to inhibit the growth of other disease-causing bacteria, including *Staphylococcus aureus* and *Escherichia coli*. Since the ethanolic extract of *A. marina* leaves contains high amounts of flavonoids and tannins, the antibacterial activity of the plant can be

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attributed to these compounds. Due to limited tests, the bactericidal or bacteriostatic status of the bioactive compounds in the A. marina leaf extract investigated in this study is difficult to ascertain. Therefore, there is a need to conduct more in-depth research in the future.

The secondary metabolites play an important role in determining the antibacterial properties of the plant extract (Normayunita et al., 2015). Flavonoids and tannins have been reported to inhibit enzyme activities and demonstrated the ability to interact with bacterial DNA, causing damage and increasing the cell walls' permeability (Sachithanandam et al., 2019). Consequently, bacterial cells rupture and lyse (Astriyani et al., 2017). In addition, phenolic compounds are also known to extract cell contents by destroying the lipids in the cell membrane of organisms (Normayunita et al., 2015). Steroid compounds, on the other hand, have been demonstrated to interact with the cell phospholipid membrane, a layer impermeable to lipophilic compounds affecting the cell integrity, changing the cell morphology, and finally making the cells brittle and lysis (Komalasari et al., 2021).

Regarding the diameter of the inhibition zone of the bacterial growth, the effectiveness of the bioactive compounds contained in the leaves of *A. marina* is far lower compared to that of the chloramphenicol (positive control). These could be explained by the extraction methods (Palombo and Semple, 2001; Narasimhudu and Venkata, 2012) and, to some extent, by the climatic condition (Vudhivanich, 2003).

CONCLUSION

The present study demonstrated that the ethanolic leaf extract of *A. marina* contained a high number of flavonoids and tannins, in addition to saponins, steroids, and phenols. The extracts were also shown to inhibit the growth of shrimp pathogen, *V. parahaemolyticus* at the concentration of 300 ppm, which is about 17.3% effective compared to the synthetic antibiotic, chloramphenicol. Such findings can justify further study on the characterization of bioactive compounds in different parts of the *A. marina* tree found in other regions of Indonesia. The results of the present study could also become an alternative treatment for shrimp aquaculture in addition to putting more value on the mangrove ecosystem. Further studies are needed on the efficacy and *in vivo* sub-acute and chronic toxicity of *A. marina* leaf extract in shrimp infected with *V. parahaemolyticus.*

DECLARATIONS

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Authors' contribution

Azis, Gazali Salim, Agus Indarjo designed the study, participated in performing the experiments and analyzing the parameter of data, and performing the experiments and writing the manuscript. Lukman Yudho Prakoso, Retno Hartati, Achmad Daengs GS contributed to analyzing the data and writing the manuscript. Meiryani, La Ode Muhammad Aslan, Julian Ransangan participated in performing the experiments and analyzing the data. Rozi checked and confirmed the manuscript's final editing and revision draft before submission to the journal. All authors confirmed the results, and the final version of the manuscript to publish in the present journal.

Competing interests

The authors declare that all authors have no competing interests.

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 the Turnitin program from the authors.

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Immunogenicity and Efficacy of a Bivalent Inactivated Vaccine against Rabbit Hemorrhagic Disease Virus

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ABSTRACT

Rabbit hemorrhagic disease is a fatal threat to rabbits that causes sustainability problems and substantial economic losses. The aim of the current study was to compare the immuno-enhancing effects of a bivalent inactivated rabbit hemorrhagic disease virus (RHDV) vaccine adjuvanted with Montanide with an inactivated RHDV vaccine with an aluminum hydroxide gel. Montanide incomplete seppic adjuvant 71 VG was prepared as an oil emulsion, and several batches adjuvanted with an aluminum hydroxide gel were prepared. Then, 250 New Zealand rabbits aged 6 weeks were randomly allocated to three groups. Group 1 was subjected to the bivalent inactivated RHDV adjuvanted with an aluminum hydroxide gel, Group 2 received the oil-emulsion vaccine adjuvanted with Montanide, and Group 3 was left unvaccinated as a negative control group. Efficacy was determined using a hemagglutination inhibition test, and resistance was determined using virulent RHDVa and RHDV2. The clinical signs included sudden death, nervous manifestations, aimless running, lateral recumbence, and crying before death. The mortality rates were recorded at 3 weeks, 3 months, 6 months, and 12 months after vaccination. In addition, blood samples were collected on the first day as well as 1, 2, 3, 4, 6 weeks post-vaccination (WPV), and 2, 3, 4 month post-vaccination (MPV) until 12 MPV. Serological analysis indicated that the bivalent inactivated RHDV oil-emulsion vaccine was more effective than the bivalent inactivated RHDV aluminum hydroxide gel vaccine, resulting in improved immune responses and longer-lasting protective immunological responses in vaccinated rabbits. The bivalent inactivated RHDV oil-emulsion vaccine was also sterile and safe and helped the protection of the rabbits against RHDVa and RHDV2, hence reducing the time and effort required during the vaccination process and reducing the levels of discomfort for the rabbits.

Keywords: Immunity, Inactivated vaccine, Oil emulsion, Rabbit hemorrhagic disease virus

INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, fatal viral disease that causes substantial losses in rabbit populations (Dalton et al., 2015). In Egypt, RHD was documented in adult rabbits in Assiut area during the winter of 1992 (Salem and El-Ballal., 1992). Rabbit hemorrhagic disease virus (RHDV) is a single-stranded, non-enveloped virus that belongs to the genus *Lagovirus* (family Caliciviridae). The current nomenclature of this virus is based on several evolutionary relationships, with *Lagovirus europaeus* being a species of *Lagovirus*. Within this species, RHDV (G1) and European brown hare syndrome virus (G2) are divided into two genogroups: G1.1a/RHDVa for the G6 RHDVa strains, G1.1b/RHDV for the classical RHDV G1 strains, G1.1c/RHDV for the classical G2 strains, and G1.1d/RHDV for the classical G3, G4, and G5 strains. G1.2/RHDV2/b is a newly proposed label for the recently described RHDV2 (Le Pendu et al., 2017).

The RHDV2 was first discovered in 2010 in France (Le Gall-Reculé et al., 2011) and later discovered in a number of governorates in Lower Egypt in 2018 and 2019 (Erfan and Shalaby, 2020; Hamida et al., 2020). Generally, RHDVa is characterized by a high mortality rate (70-90%) with a subclinical form in rabbits aged less than 6-8 weeks, whereas RHDV2 is associated with a high mortality rate (50-70%) in young rabbits but a lower rate (20-30%) in adult rabbits (Puggioni et al., 2013).

The RHDV2 was discovered in 2018 and 2019 in several governorates in Egypt. To control the spread of RHD, rabbits are vaccinated with inactivated RHDV vaccines (OIE, 2018). However, several RHD outbreaks have been reported among rabbits vaccinated by commercially available vaccines containing classical or variant strains (RHDVa). Therefore, unique RHDV was antigenically isolated and called RHDV2 (Le Gall-Reculé et al., 2011; Dalton et al., 2012). However, no cross-protective effect was observed between RHDVa and RHDV2 (Bárcena et al., 2015; OIE, 2019; Abd El-Moaty et al., 2020). According to the World Organisation for Animal Health (OIE), rabbits should be vaccinated by vaccines containing two types of RHDV (OIE, 2019).

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Montanide incomplete seppic adjuvant (ISA) is an oil-emulsion emulsifier and immuno-modulator used to produce different oil-emulsion veterinary vaccines to improve the immune response (Suckow et al., 2012). The first goal of this study was to develop a bivalent inactivated RHDV oil-emulsion vaccine (Montanide ISA 71 VG) and compare it with a vaccine containing antigenic RHDVa and RHDV2 adjuvanted with an aluminum hydroxide gel. The other goal was to evaluate the efficacy and safety of this vaccine to help control RHD outbreaks in Egypt.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Veterinary Serum and Vaccine Research Institute (VSVRI) Abbasia, Agriculture Research center, Ministry of Agriculture, Cairo, Egypt. All procedures and rabbit care steps were performed following the institutional rules for using animals in research.

Rabbit hemorrhagic disease virus

Two Egyptian RHDV strains (Giza/2006 RHDVa strain and Mahala2019/VSVRI RHDV2 strain) were provided by the VSVRI for vaccine manufacture, challenge of vaccinated rabbits, and hemagglutination inhibition (HI) test.

Rabbit hemorrhagic disease virus a

Giza/2006 is a local Egyptian strain of RHDVa (accession no. HE963222) with a titer of $10^{6.5}$ LD_{50}/mL and a hemagglutination (HA) titer of 2^{14} HA units.

Rabbit hemorrhagic disease virus 2

Mahala2019/VSVRI is a local Egyptian strain of RHDV2 (accession no. MK736667) with a titer of $10^{6.7}$ LD_{50}/mL and an HA titer of 2^{12} HA units.

Experimental rabbits

A total of 280 New Zealand susceptible male rabbits (1.5-2.0 kg) were obtained for preparation (10 rabbits, 8 weeks old), safety tests (20 rabbits, 8 weeks old), and vaccine evaluation (250 rabbits, 6 weeks old). All rabbits used were confirmed to be seronegative for RHDV through the HI test.

Preparation of vaccines

All procedures were performed in compliance with the OIE Terresterial Manual (OIE 2018) in the virology laboratory of VSVRI, Agricultural Research Center, Abbasia, Ministry of Agriculture, Cairo, Egypt. Briefly, the two viruses were first propagated in seronegative susceptible rabbits, and then the supernatants of RHDVa and RHDV2 were separately inactivated for 48 hours with formalin at a final concentration of 0.4% at 37°C. Viral inactivation was then determined by injecting five rabbits with an inactivated suspension and using two rabbits as a control group. The inactivated suspension was deemed ready for emulsification with a vaccine adjuvant if the infected rabbits demonstrated no clinical evidence of illness or fatality. Subsequently, a 2% aluminum hydroxide gel (constituting 20% of the vaccine volume) or Montanide ISA 71 VG oil emulsion was used as a solution adjuvant (constituting 70% of the preparation volume), Registered in General organization for veterinary services under registration number 899 with trade name Servac bivalent RHDV gel vaccine and number 855 with trade name Servac bivalent RHDV oil vaccine. Finally, the vaccine dose was modified to 2^{10} HA units in 0.5 mL per rabbit and subcutaneously administered in single injections (Peshev and Christova, 2003; OIE, 2018; El-Jakee et al., 2019;).

Sterility test

The two prepared vaccines were tested for sterility following standard cultivation procedures in aerobic and anaerobic bacterial and fungal growth media and then examined daily for 14 days (OIE, 2018).

Safety

To test the safety of administering an overdose of the vaccine (three doses of the inactivated vaccine), 10 seronegative rabbits were each subcutaneously injected with 1.5 mL of the vaccine. Then, at 3 weeks post-vaccination (WPV), the rabbits were monitored for any signs of illness or local response (OIE, 2018).

Experimental design

A total of 250 susceptible rabbits, aged 6 weeks, were housed in sterilized metal cages in a well-ventilated and disinfected open yard at a temperature of approximately 25°C and relative humidity of 50%. The rabbits were fed with commercial pellets (18% proteins and 14% fibers, with a total energy of 2550 kcal) purchased from Atmida (Cairo, Egypt) and allowed to drink water ad libitum. All rabbits were kept for 2 weeks before use in the experiment to be sure that they were seronegative to RHDV. The HI testing indicated that the rabbits were seronegative to both RHDV antibody strains.

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Therefore, all rabbits were divided into three groups and each group had five replicates. Group 1 (100) were subcutaneously injected with the bivalent inactivated RHDV vaccine with an aluminum hydroxide gel at a dose of 0.5 mL per rabbit (single dose), and Group 2 (100) were subcutaneously injected with the bivalent inactivated RHDV oil-emulsion vaccine at a dose of 0.5 mL per rabbit (single dose). Group 3 (50) were subcutaneously injected with normal physiological saline at a dose of 0.5 mL per rabbit as a placebo and were left unvaccinated as the control group (Kim et al., 1989).

Each group of rabbits was individually housed and kept under daily observation until the experiment was complete. Individual rabbit serum samples were obtained for all groups starting on the first day, weekly after vaccination until 4 WPV, every two weeks until 8 WPV, and then monthly until 12 WPV. Individual rabbit sera were inactivated by boiling in a water bath at 56°C for 15 minutes and stored in sterile vials at -20°C until serological analysis for specific RHDV antibodies with an HI test (Kim et al., 1989; Yuan et al., 2013).

Hemagglutination inhibition test

Each serum sample was tested for RHDVa and RHDV2 antigens twice. Before incubation at 37°C for 30 minutes, twofold serial dilutions of the serum samples were performed in 50 µL of phosphate-buffered saline, and an equal amount of virus antigen containing eight HA units were added. Then, 0.75% human red blood cells (type O) were added (50 µL) and incubated at 4°C for 1 hour. Finally, the serum dilution that demonstrated HA inhibition, as measured by mean HI $\log_2$/mL titers, was considered the endpoint (Peshev and Christova, 2003; OIE, 2018).

Virus challenge

A total of 40 rabbits from vaccinated groups (20 rabbits from each vaccinated group) and 10 rabbits from the unvaccinated group were randomly chosen for vaccine evaluation at 3 WPV, 3 months post-vaccination (MPV), 6 MPV, and 12 MPV. Then, to conduct the challenge, rabbits selected from each vaccinated group were divided into two subgroups of 10 rabbits each. Each immunized group received two types of RHDV. All rabbits in the control group received an intramuscular injection containing 1 mL of a suspension with 10^3 LD_{50} virulent RHDVa and 1 mL of a suspension with 10^3 LD_{50} virulent RHDV2 (single injection). Subsequently, all rabbits were kept under constant observation for 2 weeks, and the numbers of mortalities and postmortem lesions were documented.

RESULTS

In this study, an inactivated RHDV oil-emulsion vaccine was compared to an inactivated RHDV vaccine with an aluminum hydroxide gel. The HI tests and vaccine challenges were conducted to assess humoral immunity. Table 1 shows the estimated mean specific RHDV antibodies. Before vaccination, none of the vaccinated or unvaccinated control rabbits had RHDV particular antibodies. The humoral immune response was measured against two viruses (RHDVa and RHDV2) for each vaccine. At 1 WPV, the mean titers for specific anti-RHDV antibodies were, respectively, 2^6 and $2^{5.75}$ in the gel vaccine group and 2^4 and $2^{4.2}$ in the oil-emulsion vaccine group for RHDVa and RHDV2. The anti-RHDV antibody titers then gradually increased in the two groups, peaking at, respectively, 2^{11} and $2^{11.5}$ at 6 WPV in Group 1 and $2^{11.5}$ and $2^{11.4}$ at 5 MPV in Group 2 for RHDVa and RHDV2. The maximum mean RHDV antibody titer for RHDVa and RHDV2 was reached at 6 WPV for Group 1 and 5 MPV for Group 2.

In Group 1, the mean titers for the specific anti-RHDV antibodies for RHDVa and RHDV2 gradually increased at 1 WPV, reached their peak at 6 WPV, and then decreased but remained protective until 6 MPV; the mortality rate was 100% at 12 MPV. In Group 2, the mean titers for the specific anti-RHDV antibodies for RHDVa and RHDV2 gradually increased at 1 WPV, reached their peak at 5 MPV, and then decreased but remained protective until 12 MPV, and the mortality rate was 0% at 12 MPV.

The results of the challenge are shown in Table 2. In the current study, two local virulent strains (RHDVa and RHDV2) were used for the challenge of vaccinated rabbits with either the gel vaccine or the oil-emulsion vaccine as well as none vaccinated group at 4 intervals (3 WPV, 3 MPV, 6 MPV, 12 MPV).

These results indicated that the rabbits in both groups were completely protected against pathogenic RHDVa (10^3 LD_{50}/mL) and RHDV2 (10^3 LD_{50}/mL). As shown in Table 2, this protective effect was observed at 3 WPV, 3 MPV, and 6 MPV in Group 1 and remained until the end of the experiment (12 MPV) in Group 2.

The mortality rate was 100% at 12 MPV in the gel vaccine group, which could not resist the challenge by providing any protection. The mortality rate was 100% at 3 WPV, 3 MPV, 6 MPV, and 12 MPV in none vaccinated control group (3). The challenged rabbits of the control group could not resist the challenge by providing protection; where all rabbits died within 72 hours post challenge with specific and characteristic clinical signs and postmortem lesions of rabbit hemorrhagic disease virus and had no protection. The observed clinical signs were sudden death, nervous manifestations (ataxia, tremors, convulsions, and excitation), aimless running, lateral recumbence, paddling movement by legs, and crying before death. The characteristics of PM lesions of freshly dead rabbits were congestion and hemorrhages in the internal organs with hepatic necrosis and splenomegaly.

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Table 1. Geometric means of rabbit hemorrhagic disease virus-specific antibody titers (log_2) in the sera of vaccinated and unvaccinated rabbits

Post-vaccination period	Geometric means of RHDV HI antibody titers (log_2)					
	Group 1		Group 2		Group 3	
	RHDVa	RHDV2	RHDVa	RHDV2	RHDVa	RHDV2
Day 1	0	0	0	0	0	0
1 WPV	6	5.75	4	4.2	2*	1*
2 WPV	7.6	8	4	4.2	1	0
3 WPV	8.9	8	5.4	5	1	1
4 WPV	10.7	10.3	6.5	6	2	1
6 WPV	11	11.5	7	7.2	0	0
2 MPV	10.2	11	8	8.9	1	0
3 MPV	8.2	9	10.5	10	1	1
4 MPV	7	7.5	10.5	10	0	1
5 MPV	6.2	7	11.5	11.4	1	1
6 MPV	5	5.8	10.5	11	2	0
7 MPV	3.8	4	10	10.5	2	1
8 MPV	3.2	3.8	9	10	1	1
9 MPV	3	3	8.5	9.7	0	0
10 MPV	2.8	3	8	9	1	0
11 MPV	2.4	2.8	8	7.5	2	0
12 MPV	2	2.4	7	6.5	1	0

RHDV: Rabbit hemorrhagic disease virus, HI: Hemagglutination inhibition. RHDVa: Rabbits challenged with virulent RHDVa, RHDV2: Rabbits challenged with virulent RHDV2, WPV: Weeks post-vaccination, MPV: Months post-vaccination. Group 1 included rabbits that received the gel vaccine, Group 2 included rabbits that received the oil-emulsion vaccine, and Group 3 included the unvaccinated rabbits. *Antibody titers were non-specific and non-protective. *Protection value is considered above 2^4.

Table 2. Potency of the bivalent inactivated rabbit hemorrhagic disease virus oil-emulsion vaccine with a Montanide adjuvant and an aluminum hydroxide gel adjuvant

Post-vaccination period	Group	Challenge virus	Number of challenged rabbits	Number of protected rabbits	Number of dead rabbits	Protection level (%)
3 WPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
3 MPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
6 MPV	1	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0
12 MPV	1	RHDVa	10	0	10	0
		RHDV2	10	0	10	0
	2	RHDVa	10	10	0	100
		RHDV2	10	10	0	100
	3	RHDVa	5	0	5	0
		RHDV2	5	0	5	0

RHDV: Rabbit hemorrhagic disease virus, RHDVa: Rabbits challenged with virulent RHDVa RHDV2: Rabbits challenged with virulent RHDV2, WPV: weeks post-vaccination. MPV: Months post-vaccination. Group 1 included rabbits that received the gel vaccine, Group 2 included rabbits that received the oil-emulsion vaccine, and Group 3 had the unvaccinated rabbits.

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DISCUSSION

Rabbit hemorrhagic disease is rabbits' most common viral disease, which is associated with a high mortality rate and substantial economic losses (Dalton et al., 2015). All RHD isolates were believed to be antigenically linked until 1997, when (Capucci et al. 1998) discovered a variant with different genetic and antigenic properties, which they called RHDVa. This variant was found and isolated in Egypt in 2006 (Salman, 2007). In 2018 and 2019, another variant called RHDV2 was detected in certain Egyptian governorates, associated with substantially high mortality rates, particularly in young rabbits (Abido et al., 2020; Erfan and Shalaby, 2020). In 2019, several RHDVa variant strains were detected and verified in multiple regions in Upper Egypt, posing a threat to the population of rabbits (Abodalal et al., 2021). These findings agree with those of Mahar et al. (2018), who detected both circulating RHDVa and RHDV2 strains. Abd El-Moaty et al. (2020) reported both conventional (G1.1d/RHDV) and variant (G1.1a/RHDVa) genotypes co-exist in Egyptian rabbit populations.

Generally, RHD control can be achieved using inactivated RHDV tissue vaccines (Abido et al., 2020). Limited immunological cross-protection exists between genotypes G1.1a and G1.2 (Calvete et al., 2018). Although cross-protection immunity has been observed between classical and variant RHDVa (Read and Kirkland, 2017; Abd El-Moaty et al., 2020), both RHDVa and RHDV2 exhibit no cross-protection immunity (OIE, 2018). Therefore, a multivalent RHDV vaccine should be considered because of the limited cross-protection observed in rabbits that have received monovalent vaccines (Connor et al., 2022).

In the present study, humoral immune responses were assessed against both RHDVa and RHDV2. In previous studies, humoral immune responses were assessed against either RHDVa or RHDV2 (Montbrau et al., 2016; Abido et al., 2020) because only one monovalent RHDV vaccine was available.

According to Salman (2007), the protective value of an antibody titer is 2^4, below which the titer is considered to be a non-specific, non-protective titer. In the present study, all vaccinated rabbits demonstrated protective serum antibody responses with detectable antibody titers, whereas the unvaccinated rabbits demonstrated no detectable RHDV antibody responses (Table 1). According to the OIE (2018), inactivated adjuvant vaccines induce robust protective immunity against RHD infection during 7-10 days.

Specific anti-RHDV HI antibodies against RHDVa and RHDV2 were detected at 1 WPV, which accords with the results of Smid et al. (1991), who reported the presence of anti-RHDV HI antibodies against RHDVa at 1 WPV. The inactivated RHDV gel vaccine elicited rapid immunity in the vaccinated rabbits, as evidenced by the mean titers of specific RHDV HI antibodies at 1 WPV ranging from 2^6 to $2^{5.75}$ for RHDVa and RHDV2, respectively, in Group 1. The same results were obtained by Abodalal and Tahoon (2020). These results agree with those of Abido et al. (2020), who reported a value of 2^6 for the RHDV2 gel vaccine, but a lower value than that obtained by Salman (2007) ($2^{8.2}$) for the RHDVa gel vaccine.

Comparison of the results obtained for Group 2, which received an oil-emulsion vaccine, with the results obtained with other oil-emulsion vaccines indicated that the specific titer for vaccinated rabbits is 2^4 for RHDVa at 1 WPV, which is lower than $2^{7.7}$, and 2^5 that were reported by El-Maghraby et al. (2019). Such an increased antibody titer following vaccination with an oil-emulsion vaccine can be attributed to its low viscosity and high homogeneity (Gomes et al., 1980). These results agree with those of Peshev and Christova (2003), who also used an RHDV oil adjuvanted vaccine and detected HI antibodies ($2^{6.12}$) at 1 WPV.

At 1 WPV, the specific antibody titer for RHDV2 was found to be $2^{4.2}$, which is lower than the value obtained by Abido et al. (2020) ($2^{6.7}$). This discrepancy was because Abido et al. (2020) used a different type of adjuvant (i.e., Montanide ISA 206). These results align with those of Montbrau et al. (2016). They reported that simultaneous administration of inactivated oil classical RHDVa and inactivated oil variant RHDV2 vaccines improves immunity levels starting at 7 days after vaccination.

In Group 1, the RHDV HI antibody mean titers gradually increased, reaching $2^{8.9}$ and 2^8 for RHDVa and RHDV2, respectively, at 3 WPV. These results agree with those of Abodalal and Tahoon (2020) (who reported higher titers of $2^{10.7}$ and $2^{10.3}$) and Abido et al. (2020) (who also reported a higher RHDV2 HI antibody titer of $2^{8.9}$). As evidenced by the elevated HI titers, these concentrations stimulated a strong humoral immune response against RHDVa and RHDV2, peaking at 2^{11} and $2^{11.5}$ for RHDVa and RHDV2, respectively, at 6 WPV. However, these values decreased at 2, 3, 4, 5, and 6 MPV but remained protective until finally decreasing again at 7 MPV. These results agree with those of Abodalal and Tahoon (2020), who reported that the RHDV antibody levels gradually increased and then decreased. In the present study, the mean RHDV antibody titers in Group 1 first increased and then decreased (bell shape). However, in Group 2, the mean RHDV antibody titers gradually increased, reaching 211 and 211.5 for RHDVa and RHDV2, respectively, at 5 MPV, then gradually decreasing but remaining protective, at 2^7 and $2^{6.5}$ for RHDVa and RHDV2, respectively, at 12 MPV. These results are in line with those obtained by EL-Maghraby et al. (2019).

The results obtained in this study showed that the bivalent oil-emulsion vaccine maintained the antibody titers for a longer period of time, resulting in antibody titers of 2^7 and $2^{6.5}$ for RHDVa and RHDV2, respectively, at 12 MPV. These

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values are better than those obtained with the aluminum hydroxide gel vaccine, which may be attributed to the slow release of the oil adjuvant vaccine antigen. These results agree with those of Huang (1991), who reported that the oil-emulsion vaccine induced higher and longer-lasting antibody titers than those obtained with the RHDV aluminum hydroxide gel vaccine. In conclusion, the oil-emulsion vaccine provided a slow but long-term (1 year) immune response, whereas the gel-based vaccine (water-based) provided a fast but shorter-term (6 months) immune response.

As shown in Table 2, challenging virulent RHDVa and RHDV2 (10^3 LD_{50}/mL) resulted in 100% protection against both viruses at 3 WPV. This effect lasted until 6 MPV in Group 1 and until the end of the experiment (12 MPV) in Group 2. Mortality was observed in 100% of the unvaccinated rabbits and the vaccinated rabbits of Group 1 at 12 MPV within 72 hours. No mortality was observed in Group 2. The rabbits demonstrated no sign of illness at 12 MPV, indicating that the oil-emulsion vaccine provided 100% protection against clinical signs and mortality.

This protective effect was observed at 3 WPV and lasted until 12 MPV. These findings agree with those of Shevchenko (1994), who reported 100% protection in vaccinated rabbits challenged with virulent RHDVa at 12 MPV, and Montbrau et al. (2016), who also reported 100% protection in vaccinated rabbits challenged with a virulent RHDV2 strain 7 days after vaccination.

The results further indicated that the oil-emulsion vaccine had an adequate concentration of antigens, which helped induce a high antibody titer and provided 100% protection. These results are in line with those of Stone et al. (1983) and are also consistent with those of Salman (2007), who reported that adult rabbits with RHDV antibody titers ranging from 2^6 to 2^{13} remained clinically healthy after being inoculated with a virulent RHDV.

CONCLUSION

Inactivated RHDV oil-emulsion vaccines are superior to aluminum hydroxide gel vaccines in providing long-term immunity. They can be safely used for the active immunization of rabbits against RHDV, which is considered a threat to the Egyptian rabbit industry. The vaccination process can first be initiated with a bivalent aluminum hydroxide gel vaccine to obtain a fast response and then with a bivalent oil-emulsion vaccine to achieve long-term immunity.

DECLARATIONS

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Authors' contributions

Samah El Sayed Abodalal designed the study and prepared the vaccination batches. Samah El Sayed Abodalal and Mohamed Abdelkhalek Abdrabo performed the experimental and serological procedures. Samah El Sayed Abodalal analyzed and interpreted the data and wrote the manuscript. This manuscript content was authored, reviewed, and approved by Lamiaa Mohamed Omar, Mohamed Abdelkhalek Abdrabo, and Samah El Sayed Abodalal for publication.

Competing interests

The authors declare no competing financial or personal interests that may have influenced the research presented herein.

Ethical considerations

All authors approved the final version of this manuscript for publication. They also confirm that this study is free from any ethical concerns, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy. All data related to this study are prepared for publication in the present journal.

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Improving Bacterial, Oxidative, and Sensory Quality of Meat Steaks Using Cumin, Garlic, and Thyme Essential Oils

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ABSTRACT

The meat industry increasingly considers meat shelf life as a critical problem. Some essential oils contain antibacterial and antioxidant characteristics that help to keep meat safe. Therefore, the purpose of this study was to evaluate the preservation benefits, including antibacterial and antioxidant properties, of cumin, garlic, and thyme essential oils at 1% on chilled beef meat steaks, as well as their effects on pH, total volatile basic nitrogen (TVBN), thiobarbituric acid (TBA), and related sensory aspects (color, odor, appearance, consistency, and overall acceptability). The results of the current study showed that pretreating beef meat steaks with EOs of cumin, garlic, and thyme at a concentration of 1% effectively reduced levels of APC, coliform count, *staph aureus* count, TVBN, and TBA while extending shelf life to 12, 15, and 18 days when stored at 4°C. In terms of antibacterial and antioxidant properties, shelf life, and sensory quality on beef meat steaks, the thyme essential oil group outperformed cumin and garlic essential oils. The current study introduced an effective natural preservative alternative that could replace undesirable synthetic substances in the future while also lowering antibiotic resistance.

Keywords: Coliforms, Cumin, Garlic, Preservation, Shelf life, Thyme

INTRODUCTION

At every stage of processing, meat is contaminated with microorganisms and chemicals. It is believed that meat is the optimum environment for the growth of bacteria that cause food poisoning and spoiling since it is a perishable item with a high nitrogen content and an appropriate pH range (Alizadeh Behbahani et al., 2021). The main causes of beef spoilage are the multiplication of microbes that induce protein disintegration and the release of toxic components (Lorenzo et al., 2018). Meat spoilage is mainly caused by microbial growth, but it is also influenced by oxidation and enzymatic processes, such as the oxidation of lipids, which results in crucial changes to the meat's flavor and nutritional value (Bonilla et al., 2014; Vital et al., 2016). Furthermore, lipid oxidation not only results in organoleptic changes but is also associated with the production of dangerous chemicals, such as aldehydes (Banerjee et al., 2017).

Synthetic preservatives are used in the meat industry to reduce potential toxicity associated with microbial load and lipid oxidation processes. Natural preservatives are plant-based molecules that could extend the shelf life of numerous foods (Atarés and Chiralt, 2016). Natural preservatives, such as phenolic compounds, are gaining popularity in the food sector as effective preservatives (Shin et al., 2017). Natural antioxidants are chosen over synthetic antioxidants because they not only extend product shelf life but also protect nutritional components (Çakmakçı et al., 2014). They are also used in the pharmaceutical and cosmetic sectors (Pateiro et al., 2018). Spices and herbs commonly used in food preparation include peppers, thyme, and cumin (Hayaloglu and Farkye, 2011). Cumin is used in traditional medicine as an astringent and stimulant, as well as to alleviate diarrhea and indigestion (Alizadeh Behbahani et al., 2019). Due to its antibacterial and antioxidant effects, cumin, a plant from the Ranunculaceae family, is a valuable medication used for years (Salem, 2005). Cumin potency is greatly boosted by a plethora of vital vitamins, minerals, and volatile chemicals (Çakmakçı and Çakır, 2011). Thyme (*Thymus vulgaris* L), used as a flavoring ingredient, also has significant antibacterial potential because of its thymol composition (Burt, 2004). *Thymus vulgaris* L. possesses fragrant and therapeutic properties as a chemically polymorphic cuisine (El-Sayed and El-Sayed, 2021b); hence thyme essential oil (TEO) has been researched for its antibacterial activities (Nieto, 2020). Thyme's antibacterial efficacy against food spoilage pathogens has been studied extensively *in vitro*, but rarely in foods (Govaris et al., 2011). Garlic and its extracts are employed as antioxidants due to the presence of several chemicals, such as alliin, allyl cysteine, and allyl disulfide; they also play an essential role as antibacterial agents (Casella et al., 2013). The use of essential oils (EOs) in foods is authorized by the Food and Drug Administration (FDA) in the United States and the European Commission in Europe. Essential oils are not subject to quantitative limits because they are generally recognized as safe (GRAS) by the FDA (FDA, 2016).

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To evaluate the microbiological and biochemical deterioration of meat products, both traditional and cutting-edge measures are still applied (Luong, 2020). Some of these indicators are required to be included by food safety authorities in some nations, such Egypt, for the accurate evaluation of spoilage. Aerobic plate count (APC), coliform, and *staphylococcus aureus* count are the top microbial spoilage evaluation parameters, whereas pH, total volatile basic nitrogen, and thiobarbituric acid reactive substances (TBARS) are the best for accurate and quick evaluation of meat chemical spoilage (EOS, 2013). The hydrogen potential (pH), at some point during food preparation, processing and storage, influences enzyme reactions and promotes microbial growth. The manufacture of safe and well-preserved food benefits from pH control. Simply put, lower pH values hinder bacterial multiplication, whereas higher pH levels encourage it (Mendes, 2013). Most meat has a pH range between 5.2 to 7, with higher-quality meat often occurring between 5.7 and 6 (Barbut, 2009). Malonaldehyde (MDA), a characteristic biomarker that offers helpful information on the lipid rancidity in meat products, is measured using the thiobarbituric acid reactive substances (TBARS) assay (Tornuk et al., 2015). The third component is the organoleptic evaluation of the meat, regardless of the employed technique—panel or instrumental—because food sensory attributes are thought to have a significant role in shaping customer choices (Fernández-López et al., 2005). Changes in the acceptable aroma, taste, color, and rancid flavor are frequently linked to the development of lipids and myoglobin oxidation in fresh or cooked meat (Pires et al., 2018; Luong, 2020). Off flavors, altered textures, and altered colors associated with spoiling reactions all reduce consumer acceptance and choice (Gong et al., 2010). Therefore, one of the most important factors to take into account is the assessment of changes in the acceptable aroma, taste, color, and flavor (Luong et al., 2020). The antibacterial effectiveness of EOs in various foods has previously been studied. However, studies continue to support the concept that different EOs possess distinct antibacterial and antioxidant properties in different ecosystems. This is primarily because various bacteria in the same environments react differently to the same applied EOs. Another aspect is the variation in EOs chemical composition used in different studies as a result of plant species used, growing area, and extraction procedure (Alexopoulos et al., 2011; Eisenman et al., 2013; Bag and Chattopadhyay, 2015). Thus, more research is required to assess the vulnerability of the microbiota of particular ecosystems to various EOs and to determine whether or not they could effectively replace synthetic preservatives.

This study aimed to investigate the preservation activities of cumin, garlic, and thyme EOs (1%) on chilled beef meat steaks, as well as their impact on associated sensory aspects (color, odor, appearance, consistency, and overall acceptability).

MATERIAL AND METHODS

Fresh beef steaks weighing 4 Kg were purchased from a butcher shop in the Egyptian province of al-Qalyubia. The collected samples were kept in sterile plastic bags and in an ice container as soon as feasible before being transported to the lab.

Preparation of samples

The beef meat steaks were separated equally into four groups, with five replicates in each group (200g per replicate). The first group was established as the control, while the other three groups received interventions at the concentrations of 1% cumin EO, 1% garlic EO, and 1% thyme EO. The experiment was repeated five times for each group, with mean values calculated.

Treatment of sample with the essential oil

On the first day, each sample that was selected to receive EO pretreatment was immersed in the emulsion solution (1% pure essential oil to a final volume of 100 ml of sterile distilled water) for 15 minutes before being allowed to drain completely for 5 minutes on a clean stainless wire mesh screen. 100 cc of sterile, distilled water was used to dip the control group. Steaks that had already undergone pretreatment were individually labeled and wrapped in polyethylene bags. The experiment involved chilling storage for 18 days at 4°C (Barbosa et al., 2009).

All samples from these groups, control and treated, were evaluated for microbiological, chemical, and sensory characteristics at the start of the experiment (within 2 hours after treatment), then on a regular basis every 3 days until decomposition was observed in each group (days 1, 3, 6, 9, 12, 15, and 18).

Bacteriological examination

Determination of aerobic plate count

The aerobic plate count (APC) in beef samples was determined in the same way that it had been done for ground beef products (Sabike et al., 2015). Each sample was prepared as a 10% homogenate, followed by tenfold serial dilutions, and 1 ml from each dilution was loaded onto two separate sterile Petri dishes. The solidified inoculation plates were then incubated at 37°C for 24 hours (ISO, 2013).

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Determination of coliforms count

For the enumeration of coliforms in beef steaks following incubation on a Violet red bile agar at 37°C, ISO 4832:2006 general guidelines were employed (ISO, 2006). As specified in APC, the samples were prepared, homogenized, and decimally diluted. Plating procedures were used to transfer 1 mL aliquots of each dilution (twice) to Petri dishes.

Determination of Staphylococcus aureus count

The count of *Staphylococcus aureus* (*S. aureus*) in beef steaks was determined twice using the surface-plating method on the Baird Parker agar plate, similarly as it had been described earlier for milk (Sabike et al., 2014). One ml from each of the previously prepared serial dilutions was spread over using a sterile bent glass spreader. The plates were kept upright in the incubator for around 10 minutes or one hour until the agar absorbed the inoculums. The infected and control plates were then inverted and incubated for 48 hours at 37°C (Bennett et al., 2001).

Chemical examination

Determination of pH

Approximately 10 g of the beef flesh sample was blended with 10ml of sterilized distilled water in a blender. The homogenate was shaken continuously at room temperature for 10 minutes. An electrical pH meter was used to determine the pH value (Bye model 6020, USA). The pH meter calibration used two buffer solutions with known pH (alkaline pH 7.01, acidic pH 4.01). As a result, after adjusting the temperature correction system, the pH electrode was cleaned with neutralized water and inserted into the homogenate (Kirk and Sawyer, 1991).

Determination of total volatile basic nitrogen

Total volatile basic nitrogen (TVBN) in beef meat steaks was evaluated by distillation (Micro-Kjeldahl technique; Vapodest 30 S distillation unit, Gerhardt, Germany), as described in previous studies (Pearson, 1968; El-Bahr et al., 2021). TVN/l00g = (ml_s H_2 So_4 n 0.1 for sample – ml H_2 So_4 n 0.1 for Blank) x 14

Determination of Thiobarbituric Acid Number

The spectrophotometric method was used to determine the Thiobarbituric Acid Number (TBA) in beef meat steaks, as defined by Egyptian organization standards (EOS, 2006).

TBA value = absorbance of sample x 7.8 (malonaldehyde (mg/Kg)

Sensory evaluation

Six member panels that had received the necessary training and testing in sensory sensitivity assessed the sensory qualities of raw meat samples. Training sessions were declared complete when panelists were satisfied with the evaluation procedure and individual scores did not deviate more than one unit from the mean score. Randomly chosen representative meat samples were served in the laboratory on porcelain plates (open area). The panelists evaluated the freshness grade using a 5-point scale, with each attribute receiving a score between 1 and 5 points based on the sensory quality requirements. The qualities of color, odor, appearance, and consistency were all assessed. The beef steaks were rated as very good, good, acceptable, unacceptable, and bad in line with the overall sensory quality scores of 5, 4, 3, 2, and 1, respectively (4.5- 5: Very good, 4-4.5: Good, 3-4: Acceptable, 2-3: Unacceptable and less than 2: Bad; Fik and Leszczyńska-Fik, 2007).

Statistical analysis

The obtained data were subjected to one-way variance analysis (ANOVA) with SPSS Version 22 (SPSS Inc. Chicago, IL, USA) and are provided as means with standard errors of the mean. The statistical model includes Duncan's multiple range tests was used to compare the means of treatments. Significant differences were observed at $p < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

This study aimed to determine the antibacterial and antioxidant benefits of 1% Cumin, garlic, and thyme EOs on the shelf-life and sensory attributes of chilled beef steaks. Meat spoilage is almost generally triggered by microbial pathways, in addition to lipid oxidation, and enzyme reactions (Iulietto et al., 2015). The characterization of microbial populations using conventional culture techniques has been the gold standard for assessing food shelf life (Luong et al., 2020). The effect of cumin, garlic, and thyme EOs on the APC of beef meat steaks chilled at 4°C is shown in Table 1. There were significant variations in APC between the control group and the beef steak groups treated with 1% Cumin, garlic, and thyme EOs and chilled at 4°C ($p < 0.05$). Compared to spoiled control beef steak on day 9 of chilling, the results showed that cumin oil pretreated beef steak had a longer shelf life on day 12.

Furthermore, garlic and thyme-treated groups exhibited the best keeping quality until the 15th and 18th days of chilling, respectively. Compared to control and cumin essential oil-treated beef steaks, groups given garlic and thyme treatments were able to keep APC below the spoiling level of 10^6 cfu/g previously defined by the Egyptian standards organization (EOS (Egyptian standards), 2004; EOS, 2005; EOS (Egyptian standards), 2013) for fresh and frozen beef.

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The current study results are consistent with previous research that supports the antibacterial activity and potential of EOs to improve the shelf life of meat products (Kalemba and Kunicka, 2003; García-Díez et al., 2017; Nieto, 2020).

The reduction, as well as a prolonged increase in APC count in beef steak pretreated with EO extracts, may be attributable to Eos's ingredients that have significant antibacterial activity (Kalemba and Kunicka, 2003; García-Díez et al., 2017). The total phenolic content of EOs is among the components most closely related to biological properties, such as antibacterial and antioxidant activity, and they have been demonstrated to be effective in protecting meat products from foodborne pathogens (Daglia, 2012; Papuc et al., 2017; Pateiro et al., 2018; Stan et al., 2021). Cumin essential oils are a valuable source of 16 major bioactive chemicals, such as cumin aldehyde, γ-terpinene, p-coumaric acid and β-pinene, with antibacterial activities (Bag and Chattopadhyay, 2015; Fang et al., 2018; El-Sayed and El-Sayed, 2021a). Moreover, in vitro tests using foodborne pathogens as well as in situ tests using total bacterial count and other spoilage indicators showed antioxidant and antimicrobial activity for TEO (Ghabraie et al., 2016; Sharma et al., 2017; Radünz et al., 2020; Huang et al., 2021). The primary bioactive antioxidant and antibacterial components in TEO derive from phytochemicals such as carvacrol thymol, p-cymene, carvacrol, and γ-terpinene (Borugă et al., 2014). Oil-soluble organosulfur compounds, such as allicin, ajoenes, and allyl sulphides, are the main phytochemicals in garlic that have antibacterial properties (Bhatwalkar et al., 2021). Significant inhibitory effects, mainly thyme, shown in the current study are consistent with past research, indicating the potential for EOs application as a natural preservative in a variety of meat products (Zhang et al., 2017a; Huang et al., 2021).

Table 2 highlights the impact of cumin, garlic, and thyme EOs on the coliform levels of beef meat steaks chilled at 4°C. Coliforms are not always pathogens; yet, fecal coliforms, such as *Escherichia coli* (*E. coli*) continue to be the usual indicator organisms indicating direct or indirect feces contamination caused by poor handling and sanitation measures throughout the meat production chain (Halkman and Halkman, 2014). Compared to a high coliform level of 4.74 log in control beef steaks after the sixth day of storage, TEO was the most effective coliform inhibitor, keeping the coliform count of beef steaks refrigerated at 4°C below four log cfu/g until day 15 of storage. However, the succeeding treatments, garlic, and cumin essential oil, delayed this count back until days 9 and 12, respectively. According to previous research, Gram-negative organisms are slightly less vulnerable to the antibacterial activity of EOs than Gram-positive bacteria (Burt, 2004). Citrobacter, Enterobacter, Hafnia, Klebsiella, and *E. coli* are examples of coliforms, which comprise a significant subfamily of the *Enterobacteriaceae* family. Thyme essential oil was more potent than other EOs due to their phytochemicals being more effective under the present studied ecosystem conditions. In diverse food habitats such as horse and chicken sausage, TEO was an efficient inhibitor of Gram-negative *Enterobacteriaceae* members, such as Coliform count, *E. coli*, and *Salmonella enterica* (Sharma et al., 2017; Huang et al., 2021). Additionally, earlier research showed that EOs have a quorum-sensing inhibitive effect on specific microorganisms, particularly unfavorable ones like gram-negative bacteria, delaying their capacity to adapt to their environment (Zhang et al., 2016; Zhang et al., 2017a).

Table 3 shows the effect of thyme, cumin, and garlic EOs (1%) on naturally occurring *Staphylococci aureus* (*S. aureus*) in beef meat steaks chilled at 4°C. The results revealed that pretreatment of beef meat steaks chilled at 4°C with thyme, garlic, and cumin EOs (1%) resulted in a substantial decrease in *S. aureus* count as compared to the control group ($p > 0.05$). It was observed that thyme and garlic EOs could keep *S. aureus* counts below 4 logs until day eighteen of storage, while cumin essential oil (1%) slowed it down until day fourteen. Essential oils and phytochemicals, particularly polyphenols, have been found to suppress microbial growth and possibly even kill bacteria in various ways. Among these, EOs can interact with bacterial cell wall components and the bacterial cell membrane, destabilizing the bacterial lipid bilayer and causing bacterial death. Along with that, EOS has the potential to inhibit microbial enzymes, disrupt protein regulation, deprive bacterial cell enzymes of substrates and metal ions, and reduce a variety of microbial virulence factors (Daglia, 2012; Papuc et al., 2017; Radünz et al., 2020).

Table 1. The influence of cumin, garlic, and thyme essential oils on the aerobic plate count of beef meat steaks chilled at 4°C

Treatment / Storage time (day)	Control Count (log cfu/g)	Cumin oil 1%		Garlic oil 1%		Thyme oil 1%	
		Count (log cfu/g)	R (%)[1]	Count (cfu/g)	R (%)	Count (cfu/g)	R (%)
1	3.87 ± 0.95^{a}	3.86 ± 0.95^{a}	-	3.86 ± 0.95^{a}	-	3.86 ± 0.95^{a}	-[3]
3	5. 76 ± 1.77^{b}	4.91 ± 0.84^{c}	44.44	4.81 ± 0.4^{c}	49.74	4.58 ± 0.3^{c}	61.90
6	6.46 ± 5.6^{b}	5.54 ± 0.46^{d}	35.14	5.23 ± 0.1^{d}	47.10	4.88 ± 0.4^{e}	60.62
9	S[2]	5.76 ± 0.0.47^{d}	-	5.46 ± 0.3^{d}	--	5.26 ± 0.11^{e}	-
12	S	5.97 ± 0.5^{d}	-	5.72 ± 0.5^{d}	-	5.48 ± 0.25^{e}	-
15	S	6.41 ± 0.1^{f}	-	5.92 ± 0.5^{d}	-	5.77 ± 0.0.33^{d}	-
18	S	S	-	6.18 ± 0.1^{e}	-	5.77 ± 0.7^{f}	-

R[1] (%): Reduction percentage was calculated using following equation: $[((C^{(a)} - C^{(b)}) - (T^{(a)} - T^{(b)}))/ (C^{(a)} - C^{(b)})] \times 100$
$C^{(a)}$: Control new count (at storage day 3 or 6), $C^{(b)}$: Control initial count, $T^{(a)}$: EOs new count (at storage day 3 or 6), $T^{(b)}$: EOs initial count, S [2]: spoilage. -[3]: With the exception of the first day, this means that the control count for the tested day point became spoiled, making it difficult to calculate the reduction percentage. Mean values with different superscripts in the same rows are significantly different at $p < 0.05$. EOS (2005) established that APC of meat should not exceed 10^6/g, otherwise it is unfit for human consumption.

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Table 2. The impact of the essential oils of thyme, cumin, and garlic on the coliform of beef meat steaks chilled at 4°C

Treatment / Storage time (day)	Control Count (log cfu/g)	Cumin oil 1%		Garlic oil 1%		Thyme oil 1%	
		Count (log cfu/g)	R[1] (%)	Count (log cfu/g)	R (%)	Count (log cfu/g)	R (%*)
1	3.34 ± 0.11[a]	3.34 ± 0.11[a]		3.32 ± 0.13[a]	-	33.2 ± 0.11[a]	-[3]
3	4.23 ± 0.11[b]	3.69 ± 0.14[c]	60.67	3.57 ± 0.01[c]	71.91	3.51 ± 0.13[c]	78.65
6	4.74 ± 0.12[b]	3.90 ± 0.16[c]	60.00	3.72 ± 0.17[c]	71.43	3.66 ± 0.14[cd]	75.71
9	S[2]	3.96± 0.11[c]	-	3.84± 0.12[d]	-	3.79± 0.15[e]	-
12	S	4.26± 0.2[b]	-	3.95±0.14[c]	-	3.89± 0.03[d]	-
15	S	4.49± 0.16[d]	-	4.04± 0.11[e]	-	3.95± 0.12[f]	-
18	S	S	-	4.46± 0.16[e]	-	4.20± 0.12[g]	-

R[1] (%): Reduction percentage was calculated using following equation: [((C [(a)] - C [(b)]) – (T [(a)] - T [(b)]))/ (C [(a)] - C [(b)])] X 100.
C [(a)]: Control new count (at storage day 3 or 6), C [(b)]: Control initial count, T [(a)]: EOs new count (at storage day 3 or 6), T [(b)]: EOs initial count, S [2]: spoilage. -[3]: With the exception of the first day, this means that the control count for the tested day point became spoiled, making it difficult to calculate the reduction percentage. Mean values with different superscripts in the same rows are significantly different at $p < 0.05$. EOS (2005) established that *S. aureus* count should not exceed 10^2/g.

Table 3. The effect of essential oils of thyme, cumin, and garlic on naturally occurring *Staphylococci aureus* in beef meat steaks chilled at 4°C

Treatment / Storage time (day)	Control Count (log cfu/g)	Cumin oil 1%		Garlic oil 1%		Thyme oil 1%	
		Count (log cfu/g)	R %	Count (log cfu/g)	R %	Count (log cfu/g)	R %*
1	2.7 ± 0.11[a]	2.7 ± 0.12[a]	-	2.7 ± 0.11[a]	-	2.6 ± 0.12[a]	-[3]
3	3.61 ± 0.2[a]	3.40 ± 0.13[a]	23.08	3.28 ± 0.12[a]	36.26	3.15 ± 0.14[a]	39.56
6	4.08 ± 0.22[ab]	3.76 ± 0.15[b]	23.19	3.57 ± 0.2[b]	36.96	3.46 ± 0.21[b]	37.68
9	S[2]	3.85 ± 0.11[ab]	-	3.69 ± 0.19[c]	-	3.6 ± 0.09[c]	-
12	S	3.95 ± 0.14[d]	-	3.79 ± 0.11[e]	-	3.71 ± 0.12[e]	-
15	S	4.04 ± 0.4[d]	-	3.9 ± 0.15[d]	-	3.8 ± 0.11[e]	-
18	S	S	-	3.95 ± 0.16[d]	-	3.88 ± 0.1[f]	-

R[1] (%): Reduction percentage was calculated using following equation: [((C [(a)] - C [(b)]) – (T [(a)] - T [(b)]))/ (C [(a)] - C [(b)])] X 100.
C [(a)]: Control new count (at storage day 3 or 6), C [(b)]: Control initial count, T [(a)]: EOs new count (at storage day 3 or 6), T [(b)]: EOs initial count, S [2]: spoilage. -[3]: With the exception of the first day, this means that the control count for the tested day point became spoiled, making it difficult to calculate the reduction percentage. Mean values with different superscripts in the same rows are significantly different at $p < 0.05$. EOS (2005) established that *S. aureus* count should not exceed 10^2/g

Table 4. The influence of thyme, cumin, and garlic essential oils on the pH of beef meat steaks chilled at 4°C

Treatment / Storage time (day)	Control	Cumin oil 1%	Garlic oil 1%	Thyme oil %
1	5.69 ± 0.01[a]	5.68 ± 0.01[a]	5.67 ± 0.01[a]	5.67 ± 0.01[a]
3	6.14 ± 0.02[a]	5.80 ± 0.01[b]	5.77 ± 0.01[b]	5.73 ± 0.01[b]
6	6.82 ± 0.02[a]	6.01 ± 0.03[b]	5.86 ± 0.01[bc]	5.79 ± 0.01[c]
9	S[1]	6.22 ± 0.02[a]	6.03 ± 0.03[b]	5.91 ± 0.02[b]
12	S	6.39 ± 0.01[a]	6.19 ± 0.02[b]	6.04 ± 0.02[b]
15	S	6.57 ± 0.03[b]	6.33 ± 0.1[c]	6.20 ± 0.01[c]
18	S	S	6.61 ± 0.03[d]	6.42 ± 0.02[e]

S[1], means spoilage. Mean values with different superscripts in the same rows are significantly different at $p < 0.05$.

Chemical quality criteria

pH

The length of time that a product will retain its good quality is known as its shelf life (Iulietto et al., 2015). The pH is one of the intrinsic elements influencing meat shelf life; the pH of beef muscle is approximately 7.0 at the moment of slaughter and drops to 5.3 to 5.8 for 18 to 40 hours. The growth of spoilage bacteria is accelerated by a higher pH (Hazards and Panel, 2016). Table 4 shows the impact of garlic, cumin, and thyme EOs (each at 1%) on the pH of beef steaks refrigerated to 4°C. Within 6 days of chilling, the pH of the control steaks rose to an adverse level of 6.82, making steaks unfit for consumption. Pretreating steaks with 1% EOs of cumin, garlic, and thyme minimized pH fluctuations and preserved their viability for 12, 15, and 18 days of chilling, respectively ($p < 0.05$). This is most likely due to antioxidant and antibacterial components of EOs preventing chemical, microbiological, and physical lipid/protein damage, hence preventing nitrogen and high alkaline volatile base generation such as ammonia creation and minimizing pH increase (Esmer et al., 2011; Badee et al., 2014). Furthermore, the susceptibility of bacteria to the antimicrobial activity of EOs tends to increase as the pH of the food decreases. At low pH, an EO's hydrophobicity rises, allowing it to dissolve more easily in the lipids of the target bacteria's cell membrane (Alizadeh Behbahani et al., 2021).

Total volatile basic nitrogen

Total volatile basic nitrogen is among the chemical indicator for assessing the microbiological quality of meat (Luong et al., 2020). Table 5 displays the effect of thyme, cumin, and garlic EOs (1% each) on the TVBN content of chilled beef meat steaks at 4°C. TVBN accumulations of more than 20 mg N/100g in raw samples, according to Egyptian

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Standards, indicate the unfitness of chilled beef products due to associated decomposition (Egyptian standards, 2013). Pretreating beef steaks with 1% EOs of cumin, garlic, and thyme reduced TVBN accumulation below the critical spoiling level and retained beef quality for 12, 15, and 18 days ($p < 0.05$). Nitrogen and high alkaline volatile base formation, such as ammonia generation, is connected with chemical, microbiological, and physical lipid/protein degradation (Luong et al., 2020). The results of this investigation support previous findings that EOs, particularly TEO, are effective at suppressing undesirable microbiological and chemical alterations like TVBN in examined beef products (Ghabraie et al., 2016; Sharma et al., 2017; Huang et al., 2021). The differences in EO composition may account for the variations in pH, TVBN, and TBA among EOs containing beef steaks (Sharma et al., 2017).

Thibarbituric acid

Lipid oxidation is a type of meat deterioration that causes meats to gradually lose sensory and nutritional quality, impacting customer acceptance. It happens when polyunsaturated fatty acids interact with reactive oxygen species, causing a cascade of secondary events that end in lipid breakdown and the development of oxidative rancidity (Amaral et al., 2018). To assess rancidity or the onset of decomposition in chilled beef meat, TBARS must be tested and must not exceed 0.9 mg/kg, or the meat will be judged unfit for consumption of EOS (Egyptian standards, 2013). The effect of 1% garlic, cumin, and thyme EOs on the oxidative stability (TBA) of chilled beef meat steaks at 4°C is shown in Table 6. Some oils are more antimicrobial than others when used on meat. High-fat content appears to reduce the efficacy of EOs in meat products significantly. Depending on the type of oil applied, the amount of fat provides varying degrees of protection to the bacterial cells (Burt, 2004). When compared to Egyptian TBARS standards (Egyptian standards, 2013), the results demonstrate that the control chilled beef meat steaks are unsatisfactory on day 6 of chilling.

In contrast, those treated with 1% cumin, garlic, and thyme EOs remain acceptable till days 12, 15, and 18, respectively. The strong antioxidant activity of EOs derived from their phenolic diterpene structure, which inhibits free radical formation, might explain the lower TBRS progression in steaks treated with EOs. A few plant phenolic compounds also can act as singlet O2 quenchers and metal chelators (Shahidi et al., 1992). Nonetheless, the organosulfur compounds alliin, allylsulfide, propylsulfide, and diallylsulfide, as well as tert-butylhydroquinone (TBHQ), which are active in garlic, are responsible for its potent antioxidant and prevent malonaldehyde generation (Dewi et al., 2010; GHEISARI and RANJBAR, 2012; Nurwantoro et al., 2015).

Sensory evaluation

Sensory assessments conducted by a panel of multiple members were recognized as the most often utilised methods for assessing meat products' organoleptic quality and spoilage judgments (Luong et al., 2020). Table 7 shows the sensory characteristics of control and treated beef steak with cumin, garlic oil, and thyme EOs at a concentration of 1% stored at 4°C. According to sensory analysis, beef steaks treated with cumin, garlic oil, and thyme EOs at a concentration of 1% considerably maintained their acceptable quality until days 9, 12, and 18 of chilling, respectively ($p \leq 0.05$). In contrast, control beef steaks had poor sensory features on day 6 of chilling. These findings reflect the effects of applied EOs' antibacterial and antioxidant properties on the sensory quality of beef steaks (Sirocchi et al., 2017). Changes in lipid and pigment oxidation, as well as fatty liberation, are considered to be the primary variables influencing product appearance and flavor scores during storage (Sharma et al., 2015). In some situations, natural preservatives may alter the original flavor of the product, which is unsatisfactory to some consumers (Zhang et al., 2017b). Fortunately, the organoleptic influence would have the least detrimental effects on foods that are frequently treated with herbs, spices, or other seasonings. Nevertheless, specific EO components, such as 0.3% TEO can also give food a unique flavor (Boskovic et al., 2017), with some of them being recognized as food flavorings (Burt, 2004). Thyme oil up to 0.9 % had no negative impacts on the flavor or appearance of a coating for cooked shrimp, but when applied at 1.8%, the shrimp were much less appealing (Ouattara et al., 2001).

Table 5. The effect of thyme, cumin, and garlic essential oils on the total volatile basic nitrogen of chilled beef meat steaks at 4°C

Storage time (day) \ Treatment	Control (mg/100g)	Cumin oil 1% (mg/100g)	Garlic oil 1% (mg/100g)	Thyme oil 1% (mg/100g)
1	2.07 ± 0.09^{a}	2.03 ± 0.09^{a}	1.97 ± 0.09^{a}	1.94 ± 0.09^{a}
3	12.95 ± 0.78^{b}	5.76 ± 0.12^{c}	4.92 ± 0.11^{c}	4.08 ± 0.09^{c}
6	27.61 ± 1.10^{d}	9.21 ± 0.53^{e}	7.57 ± 0.29^{e}	6.39 ± 0.22^{e}
9	S	14.56 ± 0.69^{f}	11.84 ± 0.47^{g}	9.72 ± 0.41^{h}
12	S	18.17 ± 0.80^{de}	15.26 ± 0.52^{f}	12.98 ± 0.59^{g}
15	S	23.64 ± 0.96^{g}	19.40 ± 0.71^{h}	16.05 ± 0.63^{i}
18	S	S	24.15 ± 1.04^{g}	19.61 ± 0.65^{h}

S^{1} means spoilage. Mean values with different superscripts in the same rows are significantly different at ($p < 0.05$).

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Table 6. The impact of garlic, cumin, and thyme essential oils on the oxidative stability of chilled beef meat steaks at 4°C

Storage time (day) \ Treatment	Control (mg/Kg)	Cumin oil 1% (mg/Kg)	Garlic oil 1% (mg/Kg)	Thyme oil 1% (mg/Kg)
1	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}
3	0.57 ± 0.04^{b}	0.18 ± 0.02^{c}	0.15 ± 0.01^{c}	0.13 ± 0.01^{c}
6	1.19 ± 0.06^{e}	0.47 ± 0.03^{d}	0.31 ± 0.01^{f}	0.24 ± 0.01^{f}
9	S^{1}	0.60 ± 0.05^{d}	0.44 ± 0.03^{df}	0.32 ± 0.02^{df}
12	S	0.79 ± 0.05^{g}	0.58 ± 0.03^{f}	0.40 ± 0.03^{df}
15	S	1.05 ± 0.08^{e}	0.81 ± 0.07^{g}	0.67 ± 0.04^{h}
18	S	S	0.98 ± 0.06^{i}	0.83 ± 0.05^{g}

S^{1} means spoilage. Mean values with different superscripts in the same rows are significantly different at ($p < 0.05$

Table 7. Sensory characteristics of control and treated beef steak with cumin oil, garlic oil, and thyme oil at a concentration of 1% stored at 4°C

Storage time (day) \ Trait	Color (5)	Odor (5)	Appearance (5)	Consistency (5)	Overall (5)	Grade
Control						
1	4.8	4.7	4.8	4.8	4.8	Very good
3	3.5	2.9	3.1	3.3	3.2	Acceptable
6	1.8	1.4	1.5	1.4	1.5	Bad
9	S^{1}	S	S	S	S	Spoiled
12	S	S	S	S	S	Spoiled
15	S	S	S	S	S	Spoiled
18	S	S	S	S	S	Spoiled
Cumin oil 1%						
1	4.9	4.8	4.9	4.8	4.9	Very good
3	4.0	4.0	4.2	4.0	4.0	Good
6	3.6	3.8	3.8	3.6	3.7	Acceptable
9	3.4	3.2	3.1	3.1	3.3	Acceptable
12	2.4	1.8	2.4	2.2	2.2	Unacceptable
15	S	S	S	S	S	Spoiled
18	S	S	S	S	S	Spoiled
Garlic oil 1%						
1	4.9	5	4.9	4.8	4.9	Very good
3	4.4	4.0	4.2	4.6	4.3	Good
6	4.0	3.8	4.0	4.2	4.0	Good
9	3.4	3.2	3.6	3.6	3.5	Acceptable
12	3.2	2.8	3.4	3.2	3.2	Acceptable
15	2.6	2.4	2.6	2.4	2.5	Unacceptable
18	S	S	S	S	S	Spoiled
Thyme oil 1%						
1	4.8	5	5	4.8	4.9	Very good
3	4.6	4.8	4.5	4.7	4.6	Very good
6	4.2	4.4	4.2	4.4	4.3	Good
9	4.0	4.2	4.2	4.0	4.1	Good
12	3.8	3.8	4.0	3.6	3.8	Acceptable
15	3.4	3.4	3.6	3.2	3.4	Acceptable
18	3.0	3.2	3.2	3.0	3.1	Acceptable

4.5- 5: Very good, 4-4.5: Good, 3-4: Acceptable, 2-3: Unacceptable, less than 2: Bad. S^{1} means spoilage.

CONCLUSION

According to the current study findings, pretreatment of beef meat steaks with 1% cumin, garlic, and thyme EOs effectively diminished APC, coliform count, staph aureus count, TVBN, and TBARS levels, increasing shelf life up to 12, 15, and 18 days under chilling 4°C. Thyme essential oils surpassed garlic and cumin essential oils in terms of antibacterial and antioxidant activities, as well as shelf life and sensory quality on beef meat steaks. The current work introduces efficient natural preservative alternatives that, in the future, could replace undesired synthetic compounds while also reducing antibiotic resistance.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Abo baker Edris and Islam Sabeq designed the plan of work, and Sabah Tawfick, wrote the manuscript. Islam Sabeq revised and confirmed the statistical results. All authors confirmed the final revised articles.

Ethical consideration

The authors checked for ethical concerns, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy.

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Comparative Analysis of One-step and Two-step Dilution on Quality of Frozen Semen in Kintamani Dogs

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ABSTRACT

Preservation of sperm by freezing allows breeding dogs that are separated over long distances. To increase the fertility of frozen and then thawed spermatozoa, they must be able to survive the process. The current study aimed to evaluate the sperm motility and DNA integrity of Kintamani dogs extended in extenders with one-step and two-step dilution techniques. Ejaculates collected from four dogs were used in the current study. The semen was divided into two equal parts and diluted with extenders using two different dilution techniques, namely One-step dilution in Tris egg yolk containing 7% glycerol, and a two-step dilution technique diluted in an initial 2:1 with an extender, containing 20% egg yolk without glycerol. The same volume of the second extender was added, including 14% glycerol. The sample was loaded into 0.25 ml straws, cooled to 4°C for 4 hours, equilibrated, and then plunged into the liquid nitrogen. The sperm motility was evaluated using Computer-Assisted Sperm Analysis (CASA), and DNA integrity was assessed using Acridine Orange (AO) stained. Results showed that the sperm motility of Kintamani dogs in extenders using two-step dilution was significantly higher compared to the one-step dilution technique. In addition, the obtained results indicated that two types of dilution steps in Kintamani dog semen were not detrimental to the sperm DNA integrity during the freezing process. In conclusion, extenders with two types of dilution techniques could maintain sperm motility above 30%, and no difference between one and two steps dilution was detected.

Keywords: Dilution techniques, DNA integrity, Egg yolk, Kintamani dog, Motility

INTRODUCTION

The Kintamani dog is a purebred dog native to Bali. The Kintamani dogs were designated as a world breed by the FCI (Federation Cynologique Internationale) with a provisional category on February 20, 2019. According to the FCI rules, the Kintamani dog will be designated as the definitive breed if the quality is maintained and the population increases in the next 10 years. If there is no development within that period, the Kintamani dog will be removed from the world list (FCI, 2022). Therefore, in the next decade, there must be an effort to increase the population and genetic quality of Kintamani dogs.

Artificial insemination (AI) is the reproductive technology that can be applied to increase the population and improve genetic quality. In artificial insemination, semen is collected from male dogs manually and inserted into the female dog's reproductive tract using a catheter and place so that the process of fusion of gametes can occur without natural mating (Dutta and Dutta, 2020). Artificial insemination is one of the assisted reproductive techniques mainly done on animals, but its application to dogs is not as advanced as in cattle. In recent decades, advances in the field of reproductive physiology in female dogs and advances in canine semen processing techniques have made this service available worldwide (Mason, 2018), which allows the union of male gamete cells with female gamete cells (Jain et al., 2015; Patel et al., 2017). Until now, there have been many successful reports of the occurrence of conceptus by artificial insemination. The occurrence of pregnancy in artificial insemination is an indication of the quality of the semen used (da Cunha et al., 2017). The basic technique and methods of artificial insemination are relatively easy and have promising prospects (Jain et al., 2015). However, frozen semen for Kintamani dogs is not available yet. In all animals, cryopreservation of semen can be used to retain and preserve fertility for genetic improvement, and to increase the breeding efficiency of important breeds (Patti et al., 2021). Therefore, it is necessary to make efforts to provide Kintamani dog frozen semen.

Kintamani dog semen has been successfully preserved using a diluent based on fresh young coconut water and Tris egg yolk diluent (Puja et al., 2018). Sperm freezing in Kintamani dog has been attempted previously by either diluting semen 1:3 in coconut water and Tris egg yolk containing 7% glycerol, the process of adding extenders to the semen vary.

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Some add extenders used one-step dilution, and others used a two-step solution. Although one-step dilution is considered optimal, no studies have evaluated the effects of the two-dilution step protocol. The advantages of one-step and two-step semen dilution techniques are still debated (Arif et al., 2020).

The one-step dilution method is more practical because the extender is mixed with semen at room temperature, packaged, equilibrated, and frozen. The two-step semen dilution method includes using the former extension without glycerol, followed by the addition of the second extender containing glycerol (García-Alvarez et al., 2010). In cattle, two-step dilution showed no significant difference compared to one-step dilution (Arif et al., 2020). However, one step dilution technique significantly improved the quality of post-thaw African wild dog semen (Van den Berghe et al., 2018). The different dilution techniques used are not yet scientifically compared. Therefore, it is necessary to evaluate the most appropriate and practical dilution techniques in an effort to improve the quality of frozen semen for Kintamani dogs. Therefore, this study was conducted to test the hypothesis that two-step dilution methods maintain the quality of the spermatozoa of Kintamani dogs after the thawing.

MATERIAL AND METHODS

The chemicals used were obtained from Sigma Chemical Company (St. Louis, MO, USA). Semen was collected from four adult male Kintamani dogs of proven fertility, aging from 2 to 3 years. The dogs used were clinically healthy. Dogs are kept separately in cages of the same size (2×4 m), fed a mixture of meat and commercial feed, and given drinking water *ad libitum*. All experimental animals were managed according to the guidelines of the Institutional Committee for the Care and Use of Experimental Animals at the Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia.

Sample collection

To obtain sufficient semen, the ejaculates were pooled from four dogs. A total of four pooled samples were collected. Semen was collected from all dogs twice a week using the manual stimulation method (Puja et al., 2019). Only samples with a minimum of 70 motility, the normal morphology of spermatozoa of ≥ 70%, and the total sperm concentration ≥ 600 × 106 were considered for this study (Hermansson et al., 2021).

Extenders

The extender used in this research were prepared fresh. The compositions used are shown in Table 1. A Tris egg yolk extender was prepared and modified according to the procedure described by Hermansson et al. (2021).

Table 1. The diluent composition of frozen semen of Kintamani dogs

Material	Amount
Tris (g)	3.025
Fructose (g)	1.25
The citric acid (g)	1.7
Egg yolk (ml)	20
Streptomycin sulfate (mg/ml)	1
Penicillin (IU/ml)	1000
Glycerol (ml)	7
Distilled water (ml)	100
pH	6.5-6.7

Sample processing

After all the semen was collected, the ejaculate was centrifuged for 6 minutes at 700 g, and the supernatant was removed. The ejaculate sample was divided into two aliquots, and each aliquot was frozen using different dilution techniques (Korochkina et al., 2014).

One-step dilution

Semen was diluted 1:1 (one volume of semen diluted in one volume of extenders) with extenders containing 7% glycerol at room temperature. Then, the diluted semen was packed in 0.25 ml straws. Furthermore, it was equilibrated at a temperature of 4°C in a refrigerator for four hours, then the freezing process was carried out (Van den Berghe et al., 2017).

Two-step dilution

Semen was diluted within an initial 2:1 (two volumes of semen diluted in one volume of extenders containing lower glycerol concentration (3% v/v glycerol) dilution at room temperature. The dilute semen was then added (3:1

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dilution; 1 volume of the pre-cooled second extender containing 11% v/v glycerol so that the final concentration of glycerol in the diluent was 7%. Then, the diluted semen is packed in 0.25 ml straws, followed by equilibration at 4°C in a refrigerator for four hours, then the freezing process is carried out (Van den Berghe et al., 2017).

Freezing techniques

In the sperm freeze procedure, the straw was placed upper tray at 6 cm height above the liquid nitrogen surface in a 50 × 33 × 35 cm Styrofoam box for 15 minutes. After that, the frozen straws were immediately immersed in liquid nitrogen at -196°C and left in LN2 for 10 minutes. The frozen straw was transferred to a liquid nitrogen container for further storage (Puja et al., 2019).

Thawing procedure

The thawing process was carried out by placing the straws into a water bath at 38.5°C for 30 seconds. The content was immediately put into a pre-warmed Leja counting chamber (Domosławska et al., 2013).

Evaluation of frozen-thawed sperm

Sperm motility was assessed using the Hamilton-Thorne Sperm Analyzer IVOS II (IMV-Technologies, Aigle, France). After thawing, an aliquot of the sperm is loaded onto a prewarm Leja slide (Leja, Nieuw-Vennep, The Netherlands). This was placed on the stage of the HTM-IVOS, the temperature of which was stabilized at 37°C. Analysis was based on capturing 30 frames at 50 Hz and counting a minimum of 200 cells. DNA integrity was evaluated using the Acridine Orange (AO) stained (Mohammed et al., 2015). For each sample, 100 cells were counted and classified with intact and denatured DNA. Cells with intact DNA integrity show green, orange, or red color if denaturation.

Statistical analysis

The percentage of sperm motility and DNA integrity of the fresh semen were analyzed using descriptive analysis. To compare differences, data regarding the percentage of sperm motility and DNA integrity of frozen semen were analyzed with Paired sample t-tests using SPSS version 25. The difference between values was considered significant when the P value was less than 0.05 (Heat, 2000). All data are presented as mean values ± standard deviation.

RESULTS

The results of macroscopic observations showed that the color of ejaculate was cloudy or milky. In this study, the characteristics of semen produced from all male dogs were good. Table 2 shows the mean total sperm number, percentage of motility, percentage of spermatozoa life, and normal sperm morphology, which are generally normal so that all semen is suitable for processing. The results also indicated that individual variation led to no significant differences in semen individual characteristics.

The motility percentage reduced significantly after the equilibration process ($p < 0.05$) in both dilution techniques. However, both types of dilution techniques maintained motility above 60% after the equilibration process (Table 3). In this study, a significant effect of the equilibration process was seen on motility immediately after equilibration ($p < 0.05$). Immediately after equilibration, the equilibration process significantly affects the motility of spermatozoa ($p < 0.05$). The motility of fresh semen was 91.5%, and 72.4% in one-step dilution after equilibrium and 75% in two-step dilution. However, after equilibration, there was no significant difference in the effect between one-step dilution and two-step dilution on motility ($p > 0.05$). The percentage of spermatozoa having intact DNA integrity after the equilibration process was still above 95% (Table 3). The analysis showed that the dilution technique did not significantly affect the percentage of DNA integrity after the equilibration process ($p > 0.05$).

The result of motility and DNA integrity post-thawing are shown in Table 4. The percentage of motility in post-thawed was 40.40 in Two-step dilution and 37.60 in one-step dilution, respectively. The mean motility significantly differed in two-step dilution compared to one-step dilution ($p < 0.05$). The mean motility of spermatozoa was significantly different between two-step dilution to one-step dilution ($p < 0.05$). After post-thawing, the average percentage of spermatozoa DNA was still above 95% (Table 4). This indicates that the dilution steps are suitable for Kintamani dog semen. The results of the analysis showed that the type of dilution technique did not significantly affect the percentage of DNA integrity ($p > 0.05$).

Table 2. Fresh semen quality of Kintamani dogs

Parameter	Value	Range
The mean total sperm number ($x10^6$)	653.33	510-750
Motility (%)	91.50	89 -94
Live sperm (%)	94.45	90-95
Normal sperm morphology (%)	96.33	96-97

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Table 3. Motility and DNA integrity percentage of spermatozoa after equilibration using two different dilution techniques in studied dogs

Dilution techniques	Motility (Percentage)	DNA Integrity (Percentage)
One-step dilution	72.40	99.00
Two-step dilution	75.00	99.00

Table 4. Motility and DNA integrity percentage of spermatozoa after thawing using two different dilution techniques in Kintamani dogs

Dilution techniques	Motility (Percentage)	DNA integrity (Percentage)
One step dilution	37.60	98.80
Two step dilution	40.40	98.60

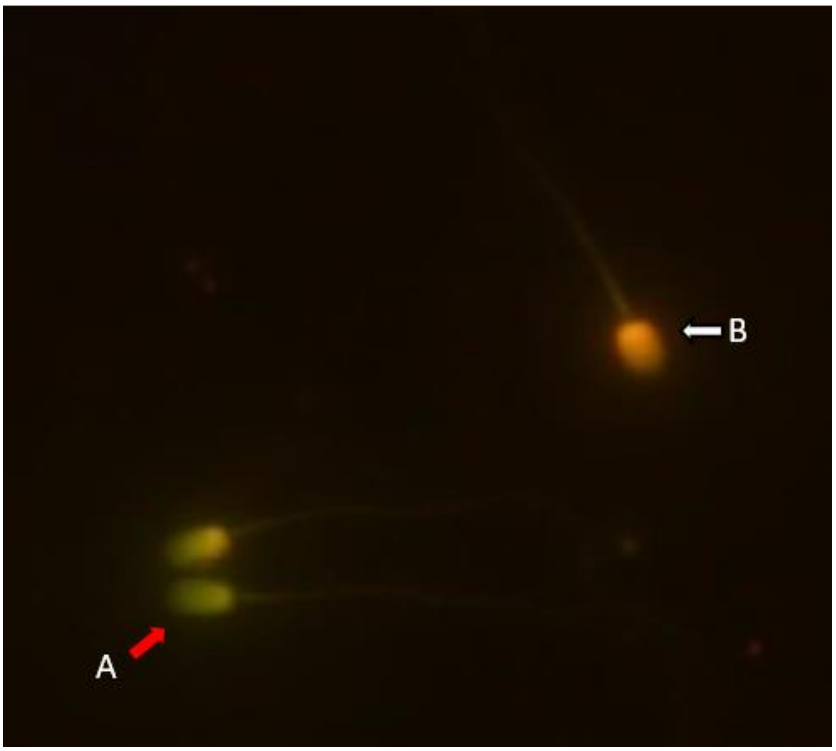

Figure 1. Sperm DNA denaturation as assessed by acridine orange test in Kintamani dog. Spermatozoa with intact DNA integrity were green (**A**: Red arrow) and denaturized were orange (**B**: white arrow)

DISCUSSION

In this study, the results indicated Kintamani dog semen could be successfully frozen with one-step or two steps dilution in Tris egg yolk extenders. Sperm motility decreased in both dilution techniques. Moreover, the DNA integrity of spermatozoa does not appear to be damaged by the freezing process. However, the extenders with one-step or two steps dilution provide sufficient post-thaw spermatozoa of Kintamani dogs for artificial insemination. This study showed that post-thawing spermatozoa motility in both dilution steps was still in the range of progressive motility that could still be used and accepted for artificial insemination in dogs. The minimal post-thawing sperm motility required for artificial insemination is around 30%. The motility between 30-65% was classified as normal spermatozoa (Hollinshead et al., 2017).

Most studies establish the ideal extenders for cryopreservation dog semen in canine reproduction. The extender was used in cattle and has been adapted for use in a dog (Martinez-Rodriguez et al., 2020). This procedure, commonly used for the production of beef bull semen straws, includes ingredients such as Tris, 7% glycerol, and 20% egg yolk (Khalil et al., 2018). Egg yolk is routinely used as the primary diluent for sperm cryopreservation in the domestic animal. Egg yolk contains lipoproteins and lecithin, which play a role in the protection and helps sperm cells in resisting against cold shock. Egg yolk provides excellent protection for semen, and can minimize the effect of cold shock that occurs during freezing and thawing (Corcini et al., 2016).

Sperm motility is one of the most important parameters in semen examination and has been the most widely used indicator for fertility in domestic dogs (Dorado et al., 2011). However, other sperm assessment, such as DNA integrity also is important because DNA integrity is associated with fertility potential (Park et al., 2018). DNA plays an important role in the function of spermatozoa that do not undergo a selection process in the female reproductive tract (Kumaresan et al., 2020). In addition, spermatozoa with broken DNA do not always show a decrease in other sperm quality parameters, but such breaks of DNA can interfere with embryonic development (Cho and Agrawal, 2018; Park et al.,

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2018). In this study, the motility of post-thawing spermatozoa was significantly different between one-step dilution and two dilution steps. The two-step dilution technique preserves the motility higher than the one-step dilution technique.

In this study, equilibration with cryoprotectants for 4 hours reduced sperm motility. According to Belala et al. (2016), the ideal equilibration time for cryopreservation of canine semen is 6 hours. Using SM extenders, the equilibration time was 3 hours (Abe et al., 2018), but based on this study, equilibration time is 4 hours in two-step dilution with a final glycerol concentration of 7%. In addition to motility, the percentage of DNA integrity is needed to determine the quality of spermatozoa. According to Agarwal and Said (2003), fertilization success is influenced by DNA integrity which is one of the parameters that must be assessed in determining the quality of spermatozoa. The AO test determines changes in DNA structure, such as DNA denaturation, which occurs in the cell nucleus. This staining technique was used to differentiate between cells with intact DNA (green staining) and cells with damaged DNA (orange). In the current study, DNA was not modified by different extenders and cryopreservation processes. DNA integrity was not significantly different in both post-thawing. Egg yolk with one-step or two-step dilutions could maintain the sperm DNA integrity of the Kintamani dog, which indicates that the type of dilution step used in this study is good for Kintamani dog semen. The results showed that the percentage of DNA integrity was not affected by the type of dilution technique used. Semen extenders can demonstrate the ability to minimize DNA fragmentation. In this study, completely denatured spermatozoa were found, which were indicated by the orange color of the sperm (Figure 1) under a fluorescence microscope as reported by Tejada et al. (1984). Considering the DNA integrity after thawing evaluated by the AO Stain and the results obtained in this study are a high percentage of sperm intact to those from Khalil et al. (2018), that observed 81% of sperm intact in frozen bull semen.

CONCLUSION

In conclusion, sperm motility was influenced by the type of dilution step technique. The motility was quite high in the two-step dilution technique. The extenders with this dilution technique could maintain motility above 30%. From the results of observations of DNA motility and integrity in both types of the dilution step technique with a final glycerol concentration of 7%, they were able to maintain motility and DNA integrity acceptable for artificial insemination during equilibration and freezing. It is, therefore, recommended to use two-step-dilution techniques for the production of frozen Kintamani dogs in Indonesia

DECLARATION

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Authors' contribution

I Nyoman Sulabda and I Ketut Puja designed the research, executed it, and wrote the manuscript. Anak Agung Gde Oka Dharmayudha assisted in collecting data and analysis. All authors read and approved the final version of the manuscript for publishing in the present journal.

Conflicts of interests

Authors have declared that no competing interests

Ethical consideration

All authors have checked the ethical issue such as plagiarism, consent to publish, misconduct, data fabrication and falsification, and redundancy.

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Canine Dystocia: The Risk Factors and Treatment Methods in Dogs of Hanoi, Vietnam

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ABSTRACT

Dystocia is a common disorder that can cause harmful health risks to bitch and puppies. The aim of the current study was to evaluate some risk factors related to canine dystocia and the application of treatment methods to 612 diagnosed cases in Gaia Pets Clinic and Resort, Hanoi, Vietnam, from December 2013 to May 2020. The investigated factors comprised age, parity and breed size, and litter size, as well as fetal sex in relation to the proportion of dystocia in female canines. Dystocia was frequently observed in female dogs aged 1-3 years, with rates of 76.1%. The highest proportion of dystocia was found in the first litter group (80.21%). The incidence of dystocia increased as the weight of the dog decreased, and it was prevalent in the small breed (61.93%). Dystocia risk decreased as the litter size increased. The interventions used in this study were medical treatment with the hormone oxytocin (1.8%), surgical management with cesarean section (86.11%), and a combination of oxytocin and cesarean section (12.09%), with the success rates of each treatment method as 100%, 98.86%, and 100%, respectively. Some risk factors, such as age, parity, breed size, and litter size identified in the present research, could be used as prognostic indicators in the veterinary practice to optimize the survival rate of female dogs and puppies.

Keywords: Age, Breed, Dystocia, Fetus sex, Litter size, Parities

INTRODUCTION

Dystocia is one of the common problems in female dog fertility leading to the maternal inability of the dog to deliver a fetus through the birth canal (Luis and Ana, 2017; Dejneka et al., 2020). Findings from 18758 bitches in the UK veterinary clinic indicated dystocia in 701 cases (3.7%, O'Neill et al., 2017). The proportion of canine dystocia is normally below 5%; however, it may reach up to 95% in French Bull, Boston Terrier, Chihuahua, and Pug dog breeds (O'Neill et al., 2017). Some studies identified breed and maternal age as risk factors for canine dystocia (Alyssa et al., 2019; Cornelius et al., 2019). In 75% of cases, dystocia was caused by the mother, while in 25% of cases, it was caused by the fetus (Pretzer, 2008). Abnormalities in canine parturition may result in the death of a dam or stillbirth.

Dystocia is a prevalent health issue seen in Vietnamese veterinary clinics and hospitals. The owner's lack of basic knowledge about canine reproduction has resulted in some complications in canine reproductive diseases (Long and Hang, 2014). Dystocia directly impacts the health of bitch, puppies' survival, and the owner's profitability. The identification of effective causes can help in management and prevention as well as reducing the likelihood of undesirable consequences.

Therefore, the current study aimed to evaluate the risk factors associated with canine dystocia in Hanoi and peripheral areas in Vietnam. The findings of the present study might be useful in improving effective treatments as well as preventive measurements in canine dystocia.

MATERIALS AND METHODS

Ethical approval

The research was approached based on Law No. 79/2015/QH13 on Veterinary Medicine 2015 of The National Assembly of the Socialist Republic of Vietnam.

Animals

A total of 612 pregnant dogs of various ages, parities, and breeds were brought into the study to diagnose the possibility of dystocia at Gaia Pets Clinic and Resort, Hanoi, Vietnam. The investigated dogs were of the breeds of Alaskan Malamute, American Bulldog, Beagle Belgian Malinois, Boston Terrier, Boxer, Bulldog, Chihuahua, Cocker, spaniel, Corgi, Dachshund, Doberman pinscher, Dogo Argentino, French Bulldog, Japanese Shiba Inu, Labrador

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Retriever, Maltese, Miniature Pinscher, Miniature Poodle, Papillon, Pekingese, Pomeranian, Pug, Rottweiler, Samoyed, Schnauzer, Shetland sheepdog, Shih Tzu, Siberian Husky, Toy Poodle, Vietnamese domestic dog, and Yorkshire. The body weights of these bitches were within the range of 1.5-57 kg. The data for the current retrospective study, including age, parities, fetal sex, fetal size, and therapeutic methods for canine dystocia were collected from December 2013 to November 2020.

Examination, diagnosis, and confirmation of dystocia

Dystocia was diagnosed in case the bitch had some parturition signs, such as nesting behavior, pollakiuria, swelling vulva, lactating, and anorexia from days 58 to 62 of gestation without giving birth (depending on fetal size and breed size). In addition, dystocia was confirmed when the bitches had expressions, such as strong contractions for more than 45-60 minutes without expulsion of a puppy, weak and infrequent contractions for more than 4-6 hours without expulsion of a puppy, and obvious radiographic abnormalities (malposition, fetal gas suggestive of fetal death, fetal oversize, fetal death, previous history of dystocia, and apparent illness or weakness of the bitch).

Classification of ages, parities, and bitches' breed size groups

The ages of bitches (by years) were specified based on their owners' records. Accordingly, bitches were categorized as they aged less than one year (G1), 1-2 years (G2), 2-3 years (G3), 3-4 years (G4), 4-5 years (G5), 5-6 years (G6), 6-7 years (G7), 7 years or older (G8). Parities were determined as normal births, excluding the previous abortions or stillbirths based on their history obtained from owners and Gaia Clinic pieces of document.

Canine breeds were divided into three groups based on the average body weight of the breeds as small-sized breeds (≤15 kg), medium-sized breeds (15-25 kg), and big-sized breeds (>25 kg) following a study by Mila et al. (2015).

Classification of litter size and fetal sex groups

Litter size was estimated by the ultrasonic scanner (2D Chison@ model ECO 1, China) and was confirmed after the bitch parturition. The litter size included alive and dead puppies. Ultrasonography was performed three times daily from 59 days of pregnancy in case of maternal compromise to obtain/confirm a suspected diagnosis and to assess fetal vitality.

Regarding the gender of puppies born, including alive and dead puppies for each parity, fetal sex was divided into three groups. The first named female fetus dominated, meaning that female fetuses were more than male fetuses in the same parity of bitch, while the second refers to male fetus dominated, where males were dominant. Finally, male and female were equated means that female and male fetuses were equal in the same parity of bitch.

Therapeutic methods of canine dystocia

The bitch's parturition was followed up by experienced veterinarians. When contractions were not regarded as normal or parturition did not progress as predicted, the oxytocin method to uterine stimulant would be applied. Before deciding to use oxytocin, veterinarians had to evaluate related fetal factors such as fetal size, fetal position, and also bitchs, such as previous breeding and birth history, the accident record or trauma to the pelvis, health, and uterine situation. Oxytocin (OXYTOCIN, Vemedim, Vietnam) was given at 2-10 IU/case (IM) with the specific dose depending on physical condition, body weight, and uterine contraction situation. The dam's cervix must be opened before the injection. Cesarean sections were performed when necessarily considered, and the veterinarian determined upon every individual case. Cesarean sections (CS) were operated on if the oxytocin therapy failed. In addition, for some dystocia high-risk breeds, CS was the preferred treatment method.

Prior to the operation, dams were shaved, disinfected, and injected with premedication; finally, the dogs underwent anesthesia. Anesthesia began with a pre-anesthesia injection with Atropine (ATROPIN, Vemedim, Vietnam) at the dose of 0.05 mg/kg (SC). After 10 minutes, anesthesia was proceeded using Tiletamine and Zolazepam (Zoletil® 50, Virbac, France) at the dose of 10 mg/kg (IV, Allerton, 2017).

Statistical analysis

The proportion test was performed to compare proportions. The software of MINITAB version 16 (Stat, Basic Statistics, 1P: 1 proportion) was used for data analysis. The probability level less than 0.05 was treated as the statistically significant difference.

RESULTS AND DISCUSSION

A total of 612 pregnant dogs were diagnosed with dystocia at Gaia Pets Clinic and Resort between 2013 and 2020. Table 1 shows that age significantly influenced dystocia in 569 bitches ($p < 0.05$). The G2 age group had the highest proportion of dystocia (39.54%), followed by the G3 age group (36.56%). Dystocia began to fade when bitches reached the age of three, with the dystocia rates in G4 to the G8 age group, being 17.78%, 4.39%, 1.76%, 1.58%, and 1.58%, respectively

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(Figure 1). However, the obtained results of the current study indicated a different association between the age of dam and dystocia rate, compared to previous studies. According to Alyssa et al. (2019), dystocia risk increased as the age of the dam increased. Furthermore, it has been found that an increase in the age of bitches (over four years) enhances the risk of dystocia by 19.2% (Forsberg and Persson, 2007), with bitches aged 3.0 to 5.9 years having 3.1 times the risk of dystocia, compared to bitches younger than three years old (O'Neill et al., 2017). Moreover, older primiparous bitches had a significantly greater frequency of single fetal pregnancies, uterine abnormalities, and extended parturition, compared to younger primiparous bitches (Münnich and Küchenmeister, 2009). The dystocia may result from the characteristics and breeding habits of Vietnamese pet owners. Apart from professional dog breeder keepers, pet owners breed their dogs once or twice, with the last breeding incorporating cesarean section and ovariohysterectomy. As a result, a small number of pregnant female dogs over the age of three were brought to the clinic with dystocia evidence. The dystocia cases referred to the clinic were observed in bitches within the age range of 1-3 years old since it is the optimum breeding time.

Table 1. The effect of age on canine dystocia in dogs of Hanoi, Vietnam

Age	Number of cases	Percentage ($p \pm \sigma_p$)
G1 (<1)	16	$2.81^{d} \pm 0.69$
G2 (1 to <2)	225	$39.54^{a} \pm 2.05$
G3 (2 to <3)	208	$36.56^{a} \pm 2.02$
G4 (3 to <4)	67	$11.78^{b} \pm 1.35$
G5 (4 to <5)	25	$4.39^{c} \pm 0.86$
G6 (5 to <6)	10	$1.76^{d} \pm 0.55$
G7 (6 to <7)	9	$1.58^{d} \pm 0.52$
G8 (≥7)	9	$1.58^{d} \pm 0.52$
	N = 569	100

[a,b,c,d;] Means within a column with different superscripts differ significantly (P < 0.05).

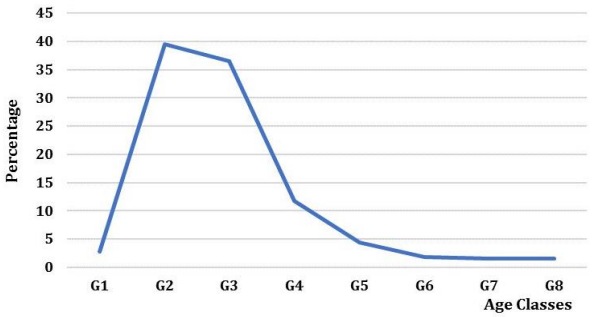

Figure 1. The relationship between age and canine dystocia G1 (Younger than a year), G2 (1-2 years), G3 (2-3 years), G4 (3-4 years), G5 (4-5 years), G6 (5-6), G7 (6-7 years), G8 (over 7 years)

Canine dystocia was also influenced by bitches' parities. The results revealed that canine dystocia is primarily focused on the first parity, which accounts for 80.21 percent of the total, and gradually decreases in the second, third, and fourth parties, which consist of 15.17%, 3.60%, and 1.03% ($p < 0.05$), respectively (Table 2). This result is consistent with previous studies that the dystocia percentage observed for bitches at the first parity was significantly higher than in other parties (Ajala and Fayemi, 2011). However, some authors concluded that there was no relationship between parity and littering in female dogs (Forsberg and Persson, 2007; Alyssa et al., 2019). Damelid and Linde-Forsberg (1994) reported that the dystocia rate of bitches with more than one parity was found to be the highest (72%), whilst the incidence for bitch in the first parity was 15%. In this study, the result given for canine dystocia of primiparous bitches was found to be the highest one because some bitches were mated at their first heat regardless of the physical underdevelopment and small pelvis which led to dystocia. In addition, female dogs had not reached the maximum fertility during the first estrus, resulting in a small litter size and large fetal size so that increasing dystocia rate. Before permitting dogs to be bred for breeding, owners should consult with veterinarians. Veterinarians also should recommend owners carefully monitor the bitch throughout late pregnancy for timely interventions in the case of the first litter.

Additionally, the incidence of dystocia is also affected by the size of the breed ($p < 0.05$, Table 3). The small-breed group had the most cases (379), accounting for 61.93% of all dystocia cases. The lowest proportion of dystocia was found in the big-sized breed group (71 cases, 11.60%). Besides, 162 cases (26.47%) of dystocia were in the medium-sized breed group. The prevalence of dystocia in the present study was mainly concentrated in some small-size and medium-size breeds. Similarly, the research of Münnich and Küchenmeister (2009) in Germany reported the highest canine dystocia rate in miniature and small breeds (59.4%). Compared to the bitch size, the fetus's relative size was larger in small breeds than in large breeds of canines (Borgea et al., 2011). In addition, the large head-to-pelvis ratio found in some certain brachycephalic breeds (Tilley and Smith, 2016) or dorso-ventrally flattened pelvic canal in Scottish terriers increased the risk of obstructive dystocia (Bergström et al., 2006), which corresponded to the dystocia risk in the present research. The majority of small and medium-size bitches that suffered from dystocia were brachycephalic breeds, such as French Bulldog, English Bulldog, and Chihuahua. Gaudet (1985) studied 128 dogs with dystocia and found that the Chihuahua, Dachshund, Pekingese, Yorkshire terrier, Miniature poodle, and Pomeranian were at a significantly higher risk than the hospital population. Pekingese and Yorkshire breeds had a lower proportion of dystocia which may be due to the unpopularity of these breeds in Vietnam.

Litter size was reported in 339 cases, with canine dystocia focusing primarily on bitches with 2-5 fetuses, accounting for 15.04% to 19.76% of all canine dystocia cases ($p < 0.05$, Table 4). Furthermore, the prevalence for cases

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with 6 or more puppies per litter revealed a downward trend, with the incidence of dystocia reducing as the number of fetuses per litter increased (Figure 2). Alyssa et al. (2019) showed that bitches with medium litter size (from 5 to 9 puppies) have the lowest incidence of experiencing dystocia, whereas small and large litter sizes have a higher risk. Stillbirth risk is increased with ≥11 puppies' litter (Borgea et al., 2011; Alyssa et al., 2019) by extending the uterus and slowing contractions during birth (Tønnessen et al., 2012). According to Borgea et al. (2011), the breed size was also affected by the number of fetuses in litters, whereby the larger the breed size, the greater the litter size. Münnich and Küchenmeister (2009) reported that female dogs with small litters (one to three puppies) had a higher prevalence of dystocia than large breeds with high litters (>8 puppies). Interbreeding between dogs of different breeds, especially large dogs (males) and small breeds (females) leads to dystocia and, consequently, absolute fetal oversize (Ajala and Fayemi, 2011). Furthermore, litter size has been identified as a risk factor for the development of uterine inertia in dogs due to the difference in SM-γ-actin gene expression between large and small litters (Egloff et al., 2020). In the present study, some cases with a medium and large litter size of more than five puppies are also positively correlated with an increased risk of dystocia because of the prolonged parturition, exhausting the mother or two amniotic from both horns coming out at the same time would reduce the ability of the dam to labor.

Table 2. Canine dystocia rate by various parities in Hanoi, Vietnam

Parities	Number of cases	Percentage ($p \pm \sigma_p$)
1	312	$80.21^{a} \pm 2.02$
2	59	$15.17^{b} \pm 1.82$
3	14	$3.60^{c} \pm 0.94$
4	4	$1.03^{d} \pm 0.51$
	N = 389	100

[a,b,c,d;] Means within a column with different superscripts differ significantly (P < 0.05).

Table 3. Canine dystocia rate by breed sizes in dogs of Hanoi, Vietnam

Breed sizes	Number of cases	Percentage ($p \pm \sigma_p$)
Small (<15kg)	379	$61.93^{a} \pm 1.96$
Medium (15-25kg)	162	$26.47^{b} \pm 1.78$
Big (>25kg)	71	$11.60^{c} \pm 1.29$
	N = 612	100

[a,b,c;] Means within a column with different superscripts differ significantly (P < 0.05).

Table 4. Percentage of dystocia by the litter size in dogs of Hanoi, Vietnam

Litter size	Number of cases	Percentage ($p \pm \sigma_p$)
1	28	$8.26^{bc} \pm 1.50$
2	67	$19.76^{a} \pm 2.16$
3	59	$17.40^{a} \pm 2.06$
4	64	$18.88^{a} \pm 2.13$
5	51	$15.04^{ab} \pm 1.94$
6	29	$8.55^{bc} \pm 1.52$
7	20	$5.90^{bc} \pm 1.28$
8	13	$3.83^{c} \pm 1.04$
>9	8	$2.36^{c} \pm 0.82$
Total	339	100

[a,b,c,d;] Means within a column with different superscripts differ significantly (P < 0.05).

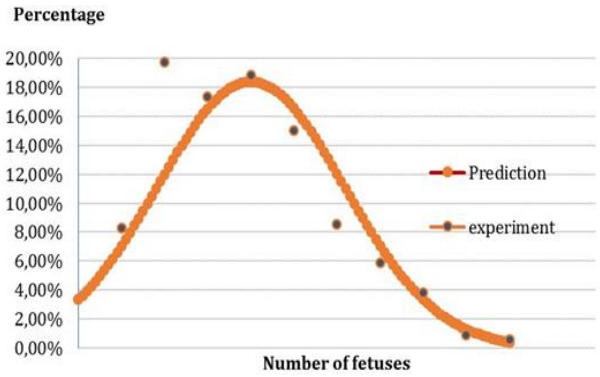

Figure 2. The correlation between the number of fetuses and the risk of dystocia in dogs

Bitches conceived in female fetuses dominated (43.75%) had a higher incidence of dystocia than bitches with male fetuses dominated (29.46%) or male and female fetuses were equated (26.79%, p < 0.05, Table 5). The dystocia rate in bitches conceived male fetus dominated was not significantly different from bitches conceived equal male and female fetuses (29.46% versus 26.79%, p > 0.05). The relationship between the gender of fetuses and canine dystocia is limited. Gram and Kowalewski (2014) revealed that the gender of puppies did not affect their birth weight. However, studies of fetal mass in horses (Elliott et al., 2009) and sheep (Gardner et al., 2007) indicated that the birth weight of male fetuses was heavier than females.

Table 6 shows that the cesarean section was the most common method (86.11%) to treat canine dystocia, followed by the combination method (12.09%) and the lowest with the oxytocin method (1.8%, Table 6). When using oxytocin techniques, surgery, or a combination of the two to treat dystocia, success rates were 100%, 98.86%, and 100%, respectively (p > 0.05). Among the CS group, there were six unsuccessful cases. In particular, one case died after the operation due to vomiting, which resulted in shock and death, two cases (one of which with a history of pneumonia) died because of the high-speed infusion of Propofol, and two cases with large litter size died because of respiratory distress, another failed case suffered from chronic kidney disease for 5-7 days after the CS, resulting in the death of the dam.

Pregnancy malpresentation, uterus problems, and prolonged parturition all resulted in significantly more litters with hypoxia and increasing stillbirth rate of the puppies (Münnich and Küchenmeister, 2009). Therefore, in order to ensure the welfare of both dam and puppies, the elective CS can be performed. The likelihood of CS did not change

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significantly between primiparous and multiparous dystocia bitches (O'Neill et al., 2019). Cesarean section is commonly used worldwide with the application rates up to approximately 50-80% (Bergström et al., 2006; Traas, 2008) with high success and benefits of proactive in time, proactive pre-and post-operative control, and pain control when puppies gain a health assessment from a qualified doctor, which avoids complications of uterine dystocia of the dam. Knowledge and understanding of the factors related to canine dystocia would assist veterinarians in choosing appropriate therapies.

Table 5. Percentage of dystocia in dogs by fetuses' sexes in Hanoi, Vietnam

Fetuses' sex in parities of bitches (*)	Number of cases (Bitches)	Percentage ($p \pm \sigma_p$)
Female fetus dominated	49	$43.75^{a} \pm 4.69$
Male fetus dominated	33	$29.46^{b} \pm 4.31$
Male and female fetuses were equated	30	$26.79^{b} \pm 4.18$
	N = 112	100

Female fetuses dominated means that female fetuses were more than male fetuses in the same parity of bitch and vice versa. Male and female fetuses were equated means that female fetuses and male fetuses were equal in the same parity of bitch. [a,b;] Means within a column with different superscripts differ significantly ($P < 0.05$).

Table 6. The percentage of dogs applied various treatment methods in Hanoi, Vietnam

Methods	Number of cases	Percentage ($p \pm \sigma_p$)	Success treatment methods (%)
Oxytocin	11	$1.80^{c} \pm 0.54$	100.00 (11/11)
Operation (Ceasarean)	527	$86.11^{a} \pm 1.40$	98.86 (521/527)
Combination method (Oxytocin + Ceasarean)	74	$12.09^{b} \pm 1.32$	100.00 (74/74)
	N = 612	100	

[a,b,c;] Means within a column with different superscripts differ significantly ($P < 0.05$). In parentheses: proportions of success in the methods.

CONCLUSION

In conclusion, this study revealed the relationship between ages, parities, breed sizes, litter sizes, fetal sex, and canine dystocia in Vietnam. The dystocia rate was highest in dogs aged 2 years and decreased as the age of bitches increased. Bitches with first parity, small-sized breed, litter size 2-5 puppies or female fetus dominated had a higher prevalence of dystocia. The prevalent therapy method for canine dystocia was a cesarean section. Applying this knowledge may help veterinarians and owners improve efficiency in the prevention and control of dystocia in dogs. The findings will support further research into the diagnosis and treatment of dystocia in dogs.

DECLARATIONS

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Authors' contribution

Su Thanh Long suggested the concept and started writing the article drafting. Su Thanh Long, Nguyen Thi Thu Hien, Phan Thi Hang, Nguyen Thi Hoai, and Pham Xuan Bach contributed to the editing and writing the final draft of the manuscript. All authors approved the analyzed data and final revised article.

Competing interests

The authors indicate that they have no conflicting interests.

Ethical consideration

Ethical issues such as plagiarism, permission to publish, misbehavior, database fabrication and/or falsification, multiple manuscript, and duplication were all examined by all authors.

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Effect of Gadolinium Orthovanadate Nanoparticles on Male Rabbits' Reproductive Performance under Oxidative Stress

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ABSTRACT

Oxidative stress as a leading factor of male infertility requires correction with modern pharmacological agents, particularly redox-active nanoparticles, to improve sperm quality and hormonal balance. The current experimental study aimed to investigate the effect of orthovanadate nanoparticles of rare earth elements, particularly Gadolinium, with pronounced redox properties on the reproductive function of male rabbits under oxidative stress. A total of 36 mature male *Hyla* rabbits were divided into three groups of intact control (n = 12) and two experimental groups, including rabbits ubder oxidative stress (n = 12), induced by the introduction of tert-Butyl hydroperoxide, and those under oxidative stress plus hydrosol of gadolinium orthovanadate nanoparticles (NPs, n = 12) intake for 14 days. There were four rabbits per three replicates in each group. Animals of all groups were kept on the same diet and had free access to water. The use of NPs led to an improvement in sperm quality indicators. There was an improvement in motility and ejaculate volume indicators (by 14.6% and 39.2%, respectively), a reduction of the content of morphologically abnormal sperm by 26.7%; normalization of sex hormones, an increase in the level of total testosterone (by 113%) with a decrease in 17-β-estradiol (by 16.5%). This sex hormones improvement led to an increase in the androgen saturation of the rabbit's body (free androgen index at the end of the experiment was 36.5%). The obtained changes were accompanied by a decrease in the oxidative load, as evidenced by a reduced content of diene conjugates and thio-barbituric acid-reactive compounds in the blood serum of rabbits by 30.4% and 26.8%, compared to the control. At the same time, there was an increase in the antioxidant potential, especially its glutathione link – the activity of glutathione peroxidase and glutathione reductase (by 42.5% and 34.2%, respectively), and the content of reduced glutathione increased by 62.3%, compared to the indicators before the introduction of NPs. The results of the study confirmed the effectiveness of using gadolinium orthovanadate NPs to correct the reproductive function of males under oxidative stress.

Keywords: Gadolinium orthovanadate, Male rabbits, Nanoparticles, Oxidative stress, Reproductive performances

INTRODUCTION

Male reproductive function is one of the most vulnerable to negative factors, both external and endogenous (Sharma et al., 2021). Infertility is noted as a consequence of unbalanced feeding, non-compliance with sanitary conditions and the mode of male use, metabolic disorders, especially vitamin and mineral ones, inflammatory, and infectious processes (Skliarov et al., 2020). The influence of such various factors is marked by an oxidative imbalance in the body of animals, which leads to a decrease in the reproductive capacity and fertilization potential of sperm (Koshevoy et al., 2021). The lack of an adequate response to the increase in the synthesis and accumulation of toxic radicals leads to oxidative stress (OS), which is the cause of male infertility in 95% of cases, while infectious processes and inflammation account for only 5% of cases (Agarwal et al., 2018). The causes of infertility cases in domestic animals are similar to human male infertility. Its main factors include Vitamin A deficiency as well as lack of Zinc and Selenium (Koshevoy et al., 2021; Skliarov et al., 2021).

Taking into account the pathogenesis of male infertility and developing the means for its correction, their pronounced antioxidant effect is of significant importance (Barik et al., 2019). In addition, nanostructures, such as nanoparticles (NPs), nanocontainers, and nanotubes, have attracted great attention in modern scientific research. Thus, NPs based on vanadates of rare earth elements, in particular Gadolinium and Yttrium, exhibit redox-active properties (Koreneva et al., 2016; Maksimchuk et al., 2021). The high antioxidant activity of these NPs makes it possible to use

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them as geroprotectors (Nikitchenko et al., 2021a). The positive effect of gadolinium orthovanadate NPs on the reproductive function of male rats with reproductopathy and experimental prostatitis has been proven (Belkina et al., 2017; Karpenko et al., 2020). Therefore, the aim of the study was to investigate the effect of gadolinium orthovanadate NPs on the dynamics of sperm quality, sex hormones, and oxidative/antioxidant balance in male rabbits affected by OS.

MATERIALS AND METHODS

Ethical approval

The present study was affirmed by the Ethics Committee of State Biotechnological University in Kharkiv, Ukraine (ethical approval No. 7-07 of May 7, 2022). All manipulations with animals were carried out in accordance with the European Convention for the protection of vertebrate animals used for experimental and scientific purposes (2006) and the General ethical principles of animal experiments adopted by the First National Congress on Bioethics (Kyiv, Ukraine, 2001).

Study design

A total of 36 adult male *Hyla* rabbits with the age of 29 weeks and a weight of 3.54 ± 0.05 kg were included in the present study. The rabbits were obtained from a private farm (Kharkiv region, Ukraine) free of charge. Animals were kept in the vivarium of the Department of Veterinary Surgery and Reproductology SBTU, Kharkiv, Ukraine. Before the start of the experiment, the animals were kept in the preparatory period without using pharmacological adaptogens. All animals were randomly divided into three experimental groups. The first group (n = 12) induced OS with tert-Butyl hydroperoxide (tBHP). The second group of animals (n = 12) received the combination of tBHP-induced OS and gadolinium orthovanadate NPs, and the third group was considered as a control group (n = 12). There were four rabbits per three replicates in each group. For the animals in the first experimental group (OS), the state of OS was simulated by administering tBHP in a dose equivalent to 1:10 LD_{50} (3.7 mg/kg body weight) for 14 days, according to Fatemi et al. (2014). After a two-week of tBHP intake, male rabbits in the second experimental group (OS+NPs) were orally administered the hydrosol of gadolinium orthovanadate NPs activated by europium at a dose of 0.05-0.10 mg/kg of live weight for 14 days. The control group received the same volume of distilled water. The concentrated feed and fresh tap water were available *ad libitum*. The rabbits were housed in a well-ventilated room at 25 ± 1°C and with a relative humidity of 55 ± 5% with a regular 12 hours light/12 hours dark cycle. The basal diet and its feeding values are indicated in Table 1.

Table 1. Composition of diet ingredients for 29-week-old male *Hyla* rabbits

Ingredient	Content
Amount of feed units (g/kg)	215
Root crops (g/kg)	190
Maize grain (g/kg)	70
Wheat bran (g/kg)	215
Bagasse (g/kg)	20
Meadow hay (g/kg)	70
Bean hay (g/kg)	60
Salt, (g/kg)	1.0
Phosphorus (g/kg)	1.0
Calcium (g/kg)	1.5
Carotene (mg/kg)	2.0
Digestible protein (per 100 g of feed in g)	14.0
Digestible energy (MJ/kg)	2.28

Gadolinium orthovanadate nanoparticles preparation

In the present research, $GdVO_4$: Eu^{3+} NPs were used. The hydrosol of gadolinium orthovanadate NPs doped with europium ions $Gd_{0.9}Eu_{0.1}VO_4$ was synthesized as previously described. To a mixture of aqueous 1 mol gadolinium chloride (0.4 ml) and 1 mol europium chloride (0.05 ml) 49.55 ml of H_2O bidi stilled water added and then dropwise: first 37.5 ml disodium ethylenediaminetetraacetic acid (EDTA) solution and second 37.5 ml of Na_3VO_4 (pH = 10.5). After vigorous stirring, the mixture was heated in a water bath under a reflux condenser for 24 hours at 100°C. The resulting colorless transparent solution under side illumination should scatter light (Tyndall's cone). The cooled solution was dialyzed against water for 24 hours to remove excess ions with a change of water every 6 hours. The NPs were characterized using a TEM-125K transmission electron microscope (Selmi, Ukraine). Nanoparticles of $GdVO_4$: Eu^{3+} have a spindle shape and are 8 × 25 nm in size (Figure 1). The concentration of NPs in the hydrosol was determined from the absorption spectra at 279 nm on a Specord 200 spectrophotometer (Jena, Germany, Klochkov et al., 2012).

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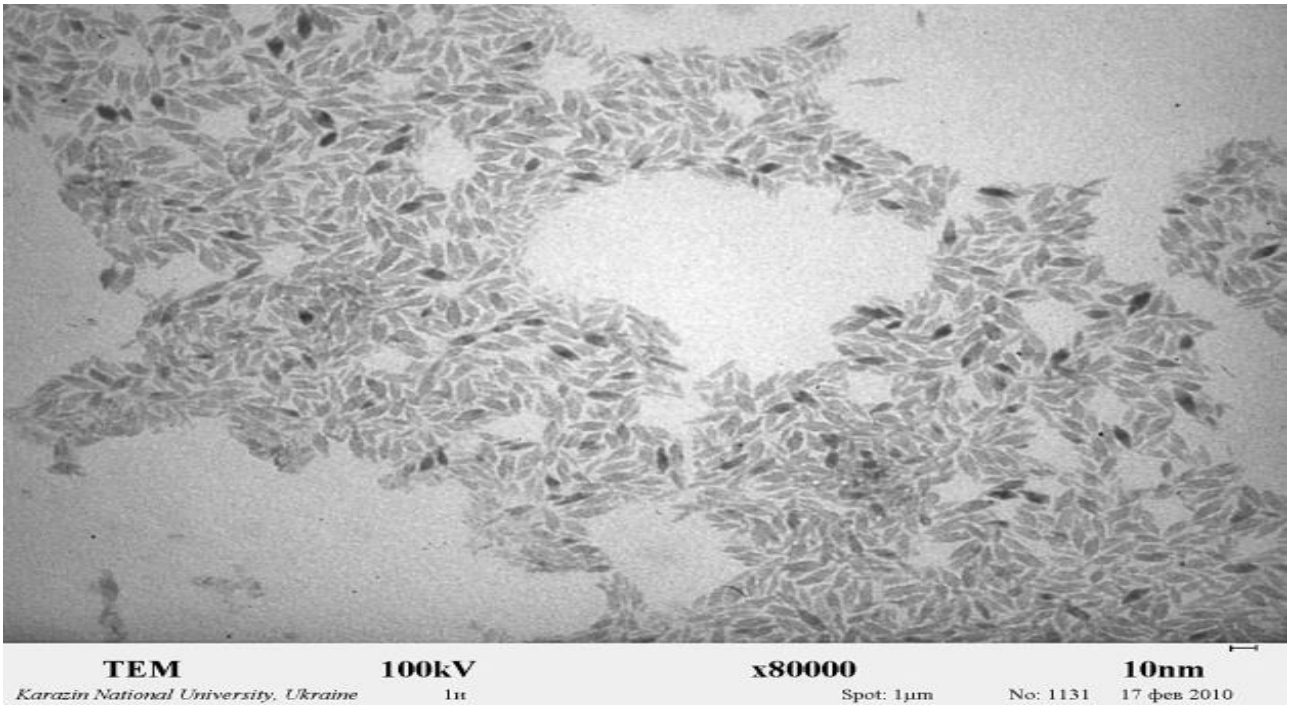

Figure 1. Electron microscopic view of the solid phase of the $GdVO_4$: Eu^{3+} colloidal solution with a size of 8×25 nm (Klochkov et al., 2012). TEM: Transmission electron microscopy

Sperm collection and evaluation

Sperm quality was assessed on days 55, 70, and 85. Males were accustomed to the artificial vagina for sperm collection prior to the experiment. Semen volume (ml), sperm motility, the number of motile sperm in the ejaculate, concentration, and percentage of sperm content with morphological abnormalities were evaluated by commonly used methods (Menon et al., 2011). The obtained ejaculates were evaluated immediately after collection. The volume of ejaculation was measured using a graduated test tube. The evaluation of live and morphologically abnormal sperm was carried out by counting 200 germ cells stained with eosin-nigrosine and expressing the obtained value as a percentage, and the number of motile sperm was counted in several fields of view using a light microscope (Zeiss, Germany) for eyepiece ×10, objective ×10. The concentration was calculated using a camera (Horyaev, Ukraine).

Blood samples collection and biochemical assay

Blood samples were taken from the lateral saphenous veins on days 15, 30, and 45 of the study (Moore et al., 2015). Pharmacological preparations for sedation and anesthesia of animals were not employed at the time of blood sampling. In the next step, 2 ml of blood samples were taken at the same time throughout the study in tubes with separating gel (BD Vacutainer®, RF).

The content of OS markers in blood serum was estimated using spectrophotometric methods in order to determine the concentrations of diene conjugates (based on the value of the molar extinction coefficient for conjugated dienes of polyunsaturated higher fatty acids wavelength $\lambda = 233$ nm) and thiobarbiturate acid-reactive compounds (based on the binding of malondialdehyde with thiobarbituric acid with the formation of a stable trimethylene complex at a wavelength of $\lambda = 532$ nm).

Antioxidants (enzymes and non-enzyme) were spectrophotometrically determined following Vlizlo (2012). Therefore, superoxide dismutase activity was calculated by the degree of reaction inhibition by the enzyme to reduce nitro blue tetrazolium in the presence of nicotinamide adenine dinucleotide and phenazine methosulfate (at $\lambda = 540$ nm). Catalase activity was determined based on the ability of hydrogen peroxide to form a stable complex with ammonium molybdate, color intensity at $\lambda = 410$ nm. Glutathione peroxidase activity (GSH-Px) was measured based on the oxidation rate of the reduced glutathione in the presence of tBHP in the color reaction with 5,5-dithiobis-2-nitrobenzoic acid (at $\lambda = 412$ nm). Glutathione reductase activity (GSH-Rd) was calculated by reducing the content of nicotinamide adenine dinucleotide phosphate at 37°C for 1 minute (at $\lambda = 340$ nm), and finally, reduced glutathione was assessed by the Butler method using Ellman's reagent (at $\lambda = 412$ nm).

Sex hormones and free androgen index

The content of Testosterone-estradiol-binding globulin (TEBG) was assessed by ELISA immunoassay to establish the dynamics of changes in the hormonal background, the level of sex hormones in the blood serum – total testosterone and 17β-estradiol with the help of standard sets of reagents ELISA Kit (LifeSpan BioSciences, USA) according to the

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instructions on the immune enzyme analyzer Stat Fax 303 plus (Awaraness Technology, USA). The Free androgen index (FAI) was calculated by taking into account the obtained data. The amount of total testosterone in blood serum was divided by the content of TEBG. The obtained values were expressed in percentages.

Statistical analysis

All data obtained during the study were processed statistically using Microsoft EXCEL software. To determine the effect of gadolinium orthovanadate nanoparticles on sperm quality, hormonal levels, and redox status of rabbits, a statistical analysis of these changes in the OS+NPs group with the OS group and the control group was performed. The obtained data from the rabbits of the control and experimental groups were analyzed using a one-way analysis of variance (ANOVA). The significant differences among means at probability were examined by Duncan's Multiple Range Test. The data in the tables were presented as mean ± standard error means (SEM). The differences between groups were considered statistically significant at $p < 0.05$.

RESULTS

Effect of $GdVO_4$: Eu^{3+} nanoparticles on sperm quality

Table 2 shows the results of the rabbit sperm quality assessment. Thus, in the rabbits of the experimental group, induced OS caused a decrease in the main characteristics of the ejaculate, in particular, the semen volume by 28.4%, the number of live sperm by 15.0%, sperm motility by 14.6%, and the concentration of sperm by 8.4% ($p < 0.05$). However, the content of morphologically abnormal cells increased significantly by 44.8%, compared to the control group ($p < 0.05$).

Positive dynamics of changes in the reproductive function of rabbits were observed using gadolinium orthovanadate NPs. For example, there was a significant increase in the volume of ejaculation by 17.0% and 34.0% on days 70 and 85, respectively, compared to the OS group ($p < 0.05$). At the same time, a significant increase in the content of live and motile sperm in the ejaculate was noted on day 70 by 15.5% and 8.7%, and on day 85 by 18.8% and 13.2%, respectively ($p < 0.05$). The concentration of germ cells underwent minor changes on days 70 and 85 of the study and increased by 3.8% and 4.7%, respectively ($p < 0.05$). A positive trend toward a significant decrease in the content of morphologically abnormal spermatozoa was noted on day 70 (15.0%). In comparison, on day 85 of the study, it was lower than that of the OS group by 25.6% ($p < 0.05$).

Table 2. Qualitative indicators of ejaculates in *Hyla* male rabbits

Experimental group (Mean±SEM) / Parameters	Control	OS	OS+NPs		
			Day 55	Day 70	Day 85
Semen volume (ml)	$0.74±0.03^{a}$	$0.53±0.02^{ab}$	$0.51±0.02^{ac}$	$0.62±0.03^{b}$	$0.71±0.02^{bc}$
Sperm concentration ($\times10^6$ sperm cell/ml)	$296.42±2.03^{a}$	$271.64±1.39^{ab}$	$272.98±1.34^{abc}$	$281.94±1.78^{bc}$	$284.40±1.94^{c}$
Sperm motility (%)	84.40	72.10	71.20	78.40	81.60
Live sperm (%)	86.30	73.40	75.60	84.80	87.20
Abnormal sperm (%)	14.30	20.70	21.00	17.60	15.40

[a,b,c] Means within the same row followed by different superscripts are significantly different ($p < 0.05$). OS: Oxidative stress, OS+NPs: Oxidative stress plus nanoparticles, SEM: Standard error means

Effect of gadolinium orthovanadate nanoparticles on sex hormones

In rabbits of the OS group, the total testosterone significantly decreased by 56.8%, while there was a significant increase in 17-β-estradiol and TEBG by 25.2% and 41.2%, respectively, compared to the control group ($p < 0.05$). As a result of such changes, the value of the free androgen index (FAI) was stable at 12.6%. The results of the effect of gadolinium orthovanadate NPs on the hormonal balance in male rabbits are shown in Table 3.

Table 3. Levels of sex hormones and the state of androgen saturation of the *Hyla* male rabbits

Experimental group (Mean±SEM) / Parameters	Control	OS	OS+NPs		
			Day 15	Day 30	Day 45
Total testosterone (nmol/l)	$4.21 ± 0.17^{a}$	$1.82 ± 0.07^{ab}$	$1.84 ± 0.06^{ac}$	$2.37 ± 0.11^{b}$	$3.89 ± 0.14^{c}$
17-β estradiol (nmol/l)	$1.31 ± 0.05^{a}$	$1.64 ± 0.06^{ab}$	$1.61 ± 0.05^{c}$	$1.58 ± 0.04$	$1.37 ± 0.03^{c}$
TEBG (nmol/l)	$10.24 ± 0.33^{a}$	$14.46 ± 0.61^{ab}$	$14.12 ± 0.58^{ac}$	$13.23 ± 0.47^{b}$	$10.67 ± 0.36^{c}$
FAI (%)	41.1	12.6	13.0	17.9	36.5

[a,b,c] Means within the same row followed by different superscripts are significantly different ($p < 0.05$). OS: Oxidative stress, OS+NPs: Oxidative stress plus nanoparticles, TEBG: Testosterone-estradiol binding globulin, FAI: Free androgen index, SEM: Standard error means

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The influence of NPs on the hormonal balance in the body of rabbits was ambiguous. For example, a significant increase of total testosterone in blood serum by 30.2% and 113% on days 30 and 45, respectively ($p < 0.05$) was accompanied by slight fluctuations in the level of 17-β-estradiol and TEBG, which tended to decrease on day 30 after the use of NPs and was lower than the indicators of the OS group by 16.5% and 26.2%, respectively on day 45 ($p < 0.05$). It should be noted that the dynamics of changes within the OS+NPs group confirm the effectiveness of the applied NPs. For example, on day 45 of the study the level of total testosterone increased by 111%, and 17-β-estradiol decreased by 14.9%, while the number of TEBG was significantly lower by 24.4% of the indicators before the introduction of NPs ($p < 0.05$).

Effect of $GdVO_4$: Eu^{3+} nanoparticles on oxidative/antioxidant balance in blood serum

The dynamics of the prooxidant-antioxidant system in rabbits are shown in Table 4. In the rabbits of the OS group, changes in the oxidative balance were observed with an increase in the oxidative load. Thus, the content of the primary products of lipoperoxidation diene conjugates (DC) was significantly higher by 57.9% of the indicators of the control group, and thiobarbituric acid-reactive compounds (TBA-RC), among which malondialdehyde with pronounced toxic properties increased by 48.3% ($p < 0.05$). In addition, a significant decrease in the antioxidant defense system activity was established in superoxide dismutase activity by 40.6% and catalase by 23.0% ($p < 0.05$). A significant decrease in the thiol-disulfide link pool reduced glutathione content by 37.2% and the activity of glutathione peroxidase and glutathione reductase by 28.7% and 20.8%, respectively ($p < 0.05$). The dynamics of antioxidant protection and peroxidation processes with NPs underwent positive changes. The number of DC decreased on days 30 (14.7%) and 45 (33.0%), respectively ($p < 0.05$), compared to the OS group. While the elimination of TBA-RC was noted (a tendency to decrease on day 30), it was 27.9% lower than the OS group on day 45 ($p < 0.05$).

The antioxidant potential under the influence of gadolinium orthovanadate NPs was normalized: an increase in Superoxide dismutase and catalase activity was noted on day 30 by 25.4% and 14.0%, respectively ($p < 0.05$), and on day 45 of the experiment by 53.3% and 26.4%, respectively ($p < 0.05$). It should be noted that a special feature of the used NPs action was the restoration of the pool of the glutathione link of antioxidant protection. On day 30 of the study, GSH content significantly increased by 37.6% ($p < 0.05$). The activity of GSH-Px and GSH-Rd on day 15 in OS + NPs group significantly increased by 31.1% and 10.7%, respectively ($p < 0.05$), compared to the OS group. On day 30 of the study, these indicators in OS + NPs group were significantly higher than the OS group as GSH, GSH-Px, and GSH-Rd increased by 66.7%, 56.8%, 32.6%, respectively ($p < 0.05$). At the same time, the obtained indicators on day 45 in OS + NPs group exceeded the data of the control group GSH-Px activity by 11.8% ($p < 0.05$), GSH-Rd activity tended to increase by 5.1%, and GSH content by 4.6%.

Table 4. Dynamics of oxidative/antioxidant status in male *Hyla* rabbits

Experimental group (Mean±SEM) / Parameters	Control	OS	OS+NPs		
			Day 15	Day 30	Day 45
DC (μmol/l)	1.21 ± 0.04^{a}	1.91 ± 0.06^{ab}	1.84 ± 0.05^{ac}	1.63 ± 0.04^{b}	1.28 ± 0.04^{bc}
TBA-RC (μmol/l)	0.87 ± 0.03^{a}	1.29 ± 0.03^{ab}	1.27 ± 0.03^{ac}	1.14 ± 0.03^{b}	0.93 ± 0.03^{bc}
Catalase activity (μmol H_2O_2/min/mg protein)	83.27 ± 1.21^{a}	64.15 ± 1.42^{ab}	63.06 ± 1.36^{abc}	73.10 ± 1.44^{b}	81.10 ± 0.89^{bc}
SOD activity (U/mgHb)	12.14 ± 0.13^{a}	7.21 ± 0.17^{ab}	7.46 ± 0.26^{abc}	9.04 ± 0.11^{b}	11.05 ± 0.09^{bc}
GSH (μmol/l)	7.12 ± 0.38^{a}	4.47 ± 0.28^{ab}	4.59 ± 0.34^{ac}	6.15 ± 0.34^{b}	7.45 ± 0.47^{bc}
GSH-Px (μmol/min×mg protein)	15.12 ± 0.24^{a}	10.78 ± 0.17^{ab}	11.86 ± 0.19^{ac}	14.13 ± 0.20^{b}	16.90 ± 0.22^{bc}
GSH-Rd (μmol/min×mg protein)	2.36 ± 0.04^{a}	1.87 ± 0.03^{ab}	1.84 ± 0.04^{ac}	2.07 ± 0.05^{b}	2.48 ± 0.03^{bc}

[a,b,c] Means within the same row followed by different superscripts are significantly different ($p < 0.05$). OS: Oxidative stress, OS+NPs: Oxidative stress plus nanoparticles, DC: Diene conjugates, TBA-RC: Thiobarbiturate acid-reactive compounds, SOD: Super oxide dismutase, GSH: Reduced glutathione, GSH-Px: Glutathione peroxidase, GSH-Rd: Glutathione reductase, SEM: Standard error means

DISCUSSION

The effectiveness of using gadolinium orthovanadate NPs, their antioxidant properties, and the effect on the reproductive function of male rabbits affected by OS is unique. Tert-Butyl hydroperoxide-induced OS caused a decrease in the main characteristics of the ejaculate (including motility and number of motile sperm in the ejaculate, concentration, and semen volume percentage of sperm content with morphological abnormalities). It is caused by a significant oxidative load on the reproductive system and a physiologically low antioxidant potential of sperm (Palani, 2018). The use of gadolinium orthovanadate NPs improved the sperm quality indicators (including sperm motility and life sperm) of male rabbits and their hormonal balance. The decrease in the number of morphologically abnormal sperm can be explained due to the antioxidant activity of the used NPs, as the decrease in their number leads to an improvement in the sexual function of rabbits (Vasicek et al., 2014). Similar changes in rabbit sperm quality were observed with the use of plant material

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(turmeric), leading to an increase in their mass, which can be economically effective, as reported by Okanlawon et al. (2020). The improvement of sperm quality indicators of spotted thistle and rosemary in rabbits was experimentally confirmed by Attia et al. (2017). Moreover, Yousef (2005) reported the possibility of using acacia leaves and their positive effect on sperm quality and hormonal balance. The obtained results showed the uniqueness of the established properties of gadolinium orthovanadate NPs, as most metal compounds in nanoform could negatively affect the physiology and metabolism of sperm. For example, the oxidizing and inflammatory effect of silver NPs on rabbit sperm cannot be eliminated even by anti-inflammatory agents and Vitamin E (Collodel et al., 2020).

Oxidative stress has a negative effect on the balance of sex hormones and reduces the amount of androgens in the male bodies due to a violation of the synthetic function of Leydig cells and the ways of regulating reproductive function, in particular, the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axis (Appasamy et al., 2007; Darbandi et al., 2018). The negative dynamics of hormonal balance were investigated by Mohammed et al. (2016). The use of boldenone undecylenate increases rabbits' growth indicators (body weight, skeletal muscle volume); however, it causes a decrease in their fertility. On the other hand, Asadi et al. (2017) confirmed an increase in changes in the balance of sex hormones (total testosterone) due to the use of molybdenum nanoparticles and their influence on the dynamics of enzyme activity in the liver (cytochrome p450) in male rats. Biologically active substances that increase rabbits' immunological resistance during cultivation are plants of the Amaranth family (Molina et al., 2018). Ghomsi et al. (2017) indicated similar changes in biochemical indicators after adding *Moringa Oleifera* leaf to the diet of rabbits, which has an antioxidant-like effect that improves the lipid profile and immune status of animals (Salem et al., 2020). Oxidative stress was characterized as a state of intensification of peroxidation processes, accumulation of active forms of Oxygen, Nitrogen, Sulfur and/or reduction of antioxidant potential (Otasevic et al., 2020). Similar changes in blood serum of male rabbits were reported by Yousef et al. (2004) for the use of isoflavonols. However, their use can lead to negative changes in sperm quality and testosterone concentration (Abo-Elsoud et al., 2019).

Some researchers used folic acid to correct male human infertility caused by chromium-VI (Yousef et al., 2006). Scarlata and O'Flaherty (2020) indicated the leading role of enzymes in the antioxidant defense system to maintain reproductive capacity. Nikitchenko et al. (2021b) confirmed the effects of gadolinium orthovanadate NPs by activating the GSH-dependent antioxidant system in male rats. Barati et al. (2020) also indicated the effectiveness of antioxidant therapy as a pathogenetic method in male infertility in humans, and drew attention to the effect of OS correction on sperm motility. Generally, the complex action of gadolinium orthovanadate NPs as fertility correctors is similar to the complex action of Zinc oxide NPs plus Thyme oil in the study by Abdel-Wareth et al. (2020). The findings indicated improved sperm quality, testosterone concentration, and nutrient digestibility in male rabbits. At the same time, similar to the NPs used, high serum antioxidant defense potential was proven in gold nanorods in the experiment by Mehanna et al. (2022).

CONCLUSION

The use of NPs of orthovanadates of rare earth elements, in particular, Gadolinium has a positive effect on the reproductive function of male rabbits by reducing the oxidative load and increasing their antioxidant potential, which leads to an increase in the volume of ejaculate and the number of motile and live spermatozoa, an increase in the concentration of germ cells in the ejaculate, and reduces effectively the content of morphologically abnormal sperm. In addition, as a result of exposure to NPs, the balance of sex hormones in the male rabbits' blood serum was normalized and the androgen saturation of their body increased. Thus, gadolinium orthovanadate nanoparticles is effective for the correction of sperm quality, the balance of sex hormones, and the dynamics of peroxidation processes in male rabbits under OS. The obtained changes indicate the presence of a complex effect of gadolinium orthovanadate NPs on the reproductive function of male rabbits under oxidative stress, and a detailed study might make it possible to substantiate the mechanism of the action of these NPs as correctors of reproductive ability. Future studies should be conducted to investigate the effect of gadolinium orthovanadate nanoparticles on the preservation of the sperm DNA structure and their fertilizing ability, as well as expanding the data on the prooxidant-antioxidant system in the tissue of the testes of male rabbits.

DECLARATIONS

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Authors' contribution

Vsevolod Koshevoy and Svitlana Naumenko designed the experiment, wrote the article, and discussed it. Pavlo Skliarov helped with the field study, collected data, and conducted the statistical analysis. Kateryna Sinyagovska and Galina Vikulina helped with laboratory analyses and tabulation of experimental data. Volodymyr Klochkov and Svitlana Yefimova helped with experiment application, and manuscript writing. All authors have read and approved the final version of the manuscript for publication in the present journal.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

Ethical issues under current regulations, including plagiarism, consent to publication, misconduct, data fabrication and/or falsification, double posting and/or submission, and redundancy, have been verified by the authors and warranted against the aforementioned violations.

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Use of Inactivated *Corynebacterium pseudotuberculosis* as an Immunostimulant with Pneumobac Vaccine

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ABSTRACT

Sheep breeders in Egypt suffer from pneumonic pasteurellosis caused by *Pasteurella trehalosi*, *Pasteurella multocida*, and *Mannheimia haemolytica*. The disease is responsible for significant economic losses in the sheep industry according to the high mortality rate and reduced carcass values. Pneumobac® is the primary vaccine in Egypt used to control pasteurellosis in sheep. Therefore, the aim of the present study was to estimate the nonspecific immune stimulating impact of *Corynebacterium pseudotuberculosis ovis* against *Pasteurella* in sheep vaccinated with Pneumobac®. Nine sheep were classified into three groups, each with three animals. The sheep in the first and second groups were inoculated with the inactivated culture of Pneumobac® and a combined inactivated culture of Pneumobac® with *Corynebacterium pseudotuberculosis ovis* bacterin, respectively. The third group was nonvaccinated and kept in control. Indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) were used to measure the humoral immune response to the produced vaccines. The results of the present study confirmed that the antibodies titer against *Pasteurella multocida* type A, D, and B6, *Pasteurella trehalosi* type T, and *Mannheimia haemolytica* type A significantly increased in sheep vaccinated with a combined vaccine (Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin), compared to those vaccinated with Pneumobac® alone. It was concluded that the addition of *Corynebacterium pseudotuberculosis ovis* bacterin to inactivated Pneumobac® vaccine could increase the immune response against pneumonic pasteurellosis.

Keywords: *Corynebacterium pseudotuberculosis*, *Pasteurella multocida*, Pasteurellosis, Pneumobac®

INTRODUCTION

Pneumonic pasteurellosis is an infectious disease caused by *Pasteurella* species that is responsible for the mortality of 25-30% and morbidity up to 50% in affected adult animals or lambs (De Alwis, 1999; James et al., 2015), especially those who have not received a sufficient colostrum amount (Kebkiba, 2021). The disease is characterized clinically by anorexia, pyrexia, occulonasal discharges, rapid shallow respiration, and pathologically by pleuritis and pneumonia (Sahay et al., 2020). Ovine pneumonia is responsible for worldwide economic loss in the sheep industry (Singh et al., 2019; Sahay et al., 2020). The microbes are commensal in the lung without causing any pathology. However, the disease appears under stress conditions, such as transportation, weaning, and diet changes (Akane et al., 2022). The disease develops in case of respiratory tract viral infection that becomes complicated by infection with *Pasteurella multocida* (*P. multocida*), *Mannheimia haemolytica* (*M. haemolytica*), and *Pasteurella trehalosi* (*P. trehalosi*) or other bacterial species, such as *Bordetella parapertussis* and *Mycoplasma ovipneumoniae* (Naglaa et al., 2019; Alarawi and Saeed, 2021). *Pasteurella and Mannheimia* bacteria are bipolar coccobacillus, gram negative, nonmotile, and facultative anaerobic bacteria (Sahay et al., 2020). Pneumonic pasteurellosis is a highly contagious disease that affects various animal species, including rodents, cattle, goats, sheep, turkeys, and rabbits. Conventional preventive measures are expensive, complex, and ineffective (Mostaan et al., 2020). In most cases of pasteurellosis, chemotherapeutic treatment is only effective for a short time before the disease reappears, as well as some drugs are toxic to human consumers; therefore the use of vaccination strategies is the best method used for control of disease in the developing nation (Ahmad et al., 2018). Recently, researchers have been directed toward producing more potent and effective vaccines (Mostaan et al., 2020). The vaccine's efficacy is determined by a variety of factors, including the amount and type of antigen used, as well as the presence of adjuvants to enhance the immunogenicity of the developed vaccine (Mandado, 2019). *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) *ovis* has shown its ability as a nonspecific immune stimulant capable of elevating sheep's resistance to artificial infection with potential pathogens, in a method similar to that produced by Bacille Calmette Guerin (BCG). The waxy elements in the *C. pseudotuberculosis* structure stimulate antibody production in the same way as *Mycobacterium* waxes associated with Freund's complete adjuvant do. Freund's

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complete adjuvant can improve the antibody response to soluble protein antigens by stimulating the innate immune system (Barakat et al., 1984; Trott et al., 2008). Pneumobac® is an inactivated oil adjuvant pasteurellosis vaccine that is the most effective used to control sheep pneumonic pasteurellosis (El-Kattan et al., 2019).

The present study aimed to evaluate *C. pseudotuberculosis* as a nonspecific immune-stimulating impact against *Pasteurella* in sheep that received the Pneumobac® vaccine.

MATERIALS AND METHODS

Ethical approval

All methods were carried out in compliance with the National Research Committee of Egypt's ethical guidelines and authorized by the Animal Care Committee (Central Laboratory for the Evaluation of Veterinary Biologics, Cairo, Egypt, Code No. 326).

Experimental animals

Nine healthy Egyptian native breeds of sheep (Rhamani) were selected after a clinical examination by an expert veterinary team from the Faculty of Veterinary Medicine and the Veterinary Serum and Vaccine Research Institute, Giza, Egypt, to confirm that all selected sheep were healthy and free from other abnormalities. The sheep were bought from Nubariah local farm for domestic animals in Giza Governorate and with an average body weight of about 25 kg and two months of age. The sheep were divided into three groups, each group containing three animals. They were housed in the farm's experimental housing (16 feet wide × 12 feet long; temperature of 61-81°F, and humidity of 30-70%) and were quarantined for latent diseases before the study. The sheep were fed on high roughage, stored hay, and low moisture grass silage. The water and food were continuously available, and the experiments were performed after two weeks of adaptation.

Bacterial strains

Pasteurella multocida type B6 standard strain, *P. multocida* types A and D, *M. haemolytica* type A, and *P. trehalosi* type T were locally isolated and identified by Prof. Eman (El-Sawah and Eman, 2010) and were used for vaccine preparation. *Corynebacterium pseudotuberculosis ovis* strain was locally isolated from Egyptian native sheep and was fully identified according to Koneman et al. (1997) and MacFaddin (2000). The *C. pseudotuberculosis ovis* strain was formalin-inactivated for the preparation of bacteria.

Synthesis of *Corynebacterium pseudotuberculosis* bacterin

Corynebacterium pseudotuberculosis ovis bacterin was performed according to the method of Auad et al. (2018). The *C. pseudotuberculosis* isolate was grown in brain heart infusion broth and cultured at 37°C in a shaking incubator for 48 hours. The bacterial cells were collected by centrifugation in a cooling centrifuge at 4000 rpm for 10 minutes. The obtained sediments were washed twice with sterile distilled water, and then the pellet was washed once with 100% acetone before being washed twice with ether and dried by air. The bacterial cells were suspended in formalin (1%) and kept overnight for complete inactivation. The inactivated bacteria were grown on brain heart infusion agar to ensure sterility.

Preparation of Pneumobac® vaccine

Formalin inactivated culture of *P. multocida* capsular biotypes A, D, and B6 contain 1×10^7 C.F.U. for each were mixed with equal volumes of formalized local isolates of *M. haemolytica* type A and *P. trehalosi* type T contains 1×10^8 C.F.U. for each. An equal amount of the above-mentioned culture was mixed using magnetic stirring (MMS-3000, Biosan) at 300 rpm according to the OIE Manual (2021) method.

Preparation of combined Pneumobac® and *Corynebacterium pseudotuberculosis ovis* bacterin vaccine

An equal amount of the inactivated Pneumobac® (VSVRI, Egypt) culture was mixed thoroughly with an equal amount of 1.5×10^8 C.F.U. of *C. pseudotuberculosis ovis* bacterin (50/50), according to the method of OIE Manual (2021).

Quality control of the produced vaccines

The produced vaccines were submitted to sterility and safety testing in accordance with the OIE Manual (2021).

Experimental design

At the age of two months, nine healthy sheep were divided into three groups (three sheep/each group). The first group was immunized with an inactivated culture of Pneumobac®, and the second group was immunized with the

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combined inactivated culture of Pneumobac® and *C. pseudotuberculosis ovis* bacterin. The third group was considered to control nonvaccinated. The vaccinated sheep were injected subcutaneously (18 G and1/4 Inch Needle length) with two doses of vaccine (1ml/dose), the one-month interval between the two doses, and blood was collected monthly until they reached 9 months of age. The collected serum samples were used to measure the immune response of vaccinated groups using enzyme linked immunosorbent assay (ELISA) and Indirect haemagglutination test (IHA).

Indirect haemagglutination test

The IHA test for detection of the *Pasteurella* antibodies was done, followed by the administration of *Pasteurella* vaccines, according to Ferede et al. (2013).

Enzyme-linked immunosorbent assay

The ELISA test was performed for detection of the Pasteurella antibodies was done followed by the administration of Pasteurella vaccines according to Takada-Iwao et al. (2007).

Statistical analysis

The statistical analysis of the resulted data was done on Minitab 14® for statistical analysis. Statistical analysis included IHA tests (tables 1 and 2), ELISA tests (tables 3 and 4), and the comparative analysis was performed by statistical T-test, Pearson correlation coefficient. Moreover, all results were assessed by one-way ANOVA with a p-value < 0.05 considered significant (Cornell, 1981).

RESULTS

Quality control of the produced vaccines

The prepared *Pasteurella* vaccines were confirmed to be safe without any morbidity or mortality when inoculated in white Swiss mice and sterile and free from any bacterial and fungal contaminants.

Indirect haemagglutination test

The humoral immune response of sheep immunized with different *Pasteurella* vaccines (Pneumobac® and combined Pneumobac® and *C. pseudotuberculosis ovis* bacterin vaccines) by using IHA. As shown in Table 1, the mean antibodies titer against *P. multocida* type A, D, and B6 in the group of sheep vaccinated with the combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) was 160, 154, and 196, respectively. The mean of antibodies titer against *P. multocida* type A, D, and B6 in the sheep group vaccinated with Pneumobac® alone were 82, 88, and 101, respectively. Moreover, the results in Table 2 demonstrated that the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T by using the IHA were 208 and 224, respectively, in the group of sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin). Moreover, the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with Pneumobac® were 116 and 114, respectively.

Table 1. Level of antibodies titer against *Pasteurella multocida* type A, D, and B6 of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by the indirect haemagglutination test

Vaccinated groups / Interval time of serum collection		Pneumobac®			Combined Pneumobac® and *Corynebacterium*			Control		
		A	D	B6	A	D	B6	A	D	B6
Pre-vaccination		2	2	2	2	2	4	0	0	0
First dose of vaccine*	First month after first vaccine	32	16	16	32	32	32	2	2	0
Booster dose of vaccine*	Second month	32	64	32	64	64	128	4	0	0
	Third month	128	128	128	256	256	512	2	0	2
	Fourth month	128	256	256	256	512	512	4	2	2
	Fifth month	256	128	256	512	256	256	2	2	0
	Sixth month	128	128	128	256	128	256	2	4	0
	Seventh month	64	64	128	128	128	128	0	0	2
	Eighth month	32	64	32	64	128	64	2	0	4
	Ninth month	16	32	32	32	32	64	0	2	0
	Overall mean	82	88	101	160	154	196	1.8	1.2	1

Number of colony count = 1×10^7 C.F.U. /ml

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Table 2. Level of antibodies titer against *Mannheimia haemolytica* type A and *Pasteurella trehalosi* type T of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by the indirect haemagglutination test

Vaccinated groups / Interval time of serum collection		Pneumobac®		Combined Pneumobac® and *Corynebacterium*		Control	
		A	T	A	T	A	T
Pre-vaccination		2	2	2	2	0	0
First dose of vaccine*	First month after first vaccine	32	16	32	64	0	2
Booster dose of vaccine*	Second month	64	64	128	128	4	2
	Third month	256	256	256	256	2	2
	Fourth month	256	256	256	512	2	2
	Fifth month	256	256	512	512	0	0
	Sixth month	128	128	256	256	0	2
	Seventh month	64	64	256	256	0	0
	Eighth month	64	64	256	128	2	2
	Ninth month	32	32	128	128	2	2
	Overall mean	116	114	208	224	1.2	1.4

Number of colony count = 1×10^8 C.F.U. /ml

Enzyme linked immunosorbent assay

As shown in Table 3, the overall mean of antibodies titer against *P. multocida* type A, D, and B6 in sheep vaccinated with combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) by using ELISA were 433, 510, and 514, respectively. The mean of antibodies titer against *P. multocida* type A, D and B6 in the group of sheep vaccinated with Pneumobac® were 314, 337, and 333, respectively. The results in Table 4 demonstrated that the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with combined vaccine was 541 and 612, respectively. While, in the sheep group that was vaccinated with Pneumobac® vaccine, the overall mean of antibodies titer against *M. haemolytica* type A and *P. trehalosi* type T were 398 and 407, respectively.

Table 3. Level of antibodies titer against *Pasteurella multocida* type A, D and B6 of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by enzyme linked immunosorbent assay

Vaccinated groups / Interval time of serum collection		Pneumobac®			Combined Pneumobac® and *Corynebacterium*			Control		
		A	D	B6	A	D	B6	A	D	B6
Pre-vaccination		10	20	10	20	10	20	0	0	0
First dose of vaccine*										
First month after first vaccine		100	250	100	180	300	290	0	0	0
Booster dose of vaccine*	Second month	205	306	202	295	466	583	0	0	0
	Third month	460	430	563	590	798	770	20	20	20
	Fourth month	505	450	710	700	765	798	0	0	0
	Fifth month	550	554	512	778	800	798	20	20	10
	Sixth month	490	500	415	655	690	798	0	0	20
	Seventh month	470	415	340	555	498	360	20	20	20
	Eighth month	200	270	210	355	415	360	20	0	0
	Ninth month	150	170	270	202	355	360	0	20	0
	Overall mean	314	337	333	433	510	514	8	8	7

Number of colony count = 1×10^7 C.F.U. /ml

Table 4. Level of antibodies titer against *Mannheimia haemolytica* type A and *Pasteurella trehalosi* type T of Rhamani sheep vaccinated with Pneumobac® and inactivated Pneumobac® combined with *Corynebacterium pseudotuberculosis* by enzyme linked immunosorbent assay

Vaccinated groups / Interval time of serum collection		Pneumobac®		Combined Pneumobac® and *Corynebacterium*		Control	
		A	T	A	T	A	T
Pre-vaccination		20	20	20	20	0	0
First dose of vaccine*							
First month after first vaccine		220	102	280	230	20	0
Booster dose of vaccine*	Second month	370	454	495	680	0	2
	Third month	453	576	590	810	20	2
	Fourth month	660	608	760	998	20	2
	Fifth month	745	650	787	790	20	0
	Sixth month	523	506	690	699	0	2
	Seventh month	440	440	655	670	0	0
	Eighth month	280	400	576	620	20	2
	Ninth month	270	318	555	600	0	2
	Overall mean	398	407	541	612	10	1.4

Number of colony count = 1×10^8 C.F.U. /ml

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DISCUSSION

For Egyptian sheep producers, pulmonary pasteurellosis is a complex disease with a morbidity of 50% and a death rate 25-30% if the animals are not treated in the early stage of infection, resulting in significant financial losses (De Alwis, 1999; El-Sawah and Eman, 2010; James et al., 2015). *Mannheimia haemolytica* and *P. multocida* are the most common organisms that cause pneumonic pasteurellosis in sheep (Taye et al., 2019). These organisms are usually found in the upper respiratory tracts of healthy animals as normal inhabitants; however, when the animal's immune system is compromised by stressors including travel, crowding, a lack of water, and concurrent viral, *Mycoplasma*, and lungworms infections, they can cause significant illness (Asfaw et al., 2022; Getnet et al., 2022). Because prevention is the most likely way to control the disease, vaccines will be of great value in protecting animals from pasteurellosis (Ismail et al., 2018). The work aimed to study the nonspecific immune-stimulating effects of *C. pseudotuberculosis ovis* bacterin against *Pasteurella* species in sheep vaccinated with Pneumobac®.

The immune response of the sheep immunized with combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) and Pneumobac® was evaluated by using an IHA as shown in Table 1, 2. Using the IHA test, the overall mean of circulating antibodies titers against *P. multocida* types A, D, and B6 in sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) showed a significant increase compared to sheep vaccinated with Pneumobac® alone ($p < 0.05$). In addition to, the overall mean of circulating antibodies against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with a combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) was significantly increased than the group of sheep vaccinated with Pneumobac® alone ($p < 0.05$). While, the result in Table 3 demonstrated the mean level of antibodies titer against *P. multocida* type A, D, and B6 in the group of sheep vaccinated with the combined vaccine (Pneumobac® and *C. pseudotuberculosis ovis* bacterin) using ELISA was significantly higher than the group of sheep vaccinated with Pneumobac® alone ($p < 0.05$). The results in Table 4 indicated that the overall mean of antibody titer against *M. haemolytica* type A and *P. trehalosi* type T in the group of sheep vaccinated with a combined vaccine was significantly increased than the group of sheep vaccinated with Pneumobac® ($p < 0.05$). The findings are in accordance with those of Barakat et al. (1984) who showed that *C. pseudotuberculosis ovis* has an immune-stimulating impact when used as an adjuvant with various antigens, including egg albumin, Food and mouth viral disease, and *Salmonella typhimurium*. In addition, the above results are inconsistent with those obtained by Eggleton et al. (1991) found that the protective efficacy of the vaccines was not improved by *C. pseudotuberculosis ovis* bacterin, but considered the toxin produced by *C. pseudotuberculosis* to be the main factor responsible for protection. In Egypt, Marwah et al. (2015) showed that the phospholipase D exotoxin of *C. pseudotuberculosis* was an effective nonspecific immune stimulant that could be used in combination with inactivated Newcastle and Mycoplasma vaccines to elicit an early, better, and longer immune response.

Freund (1956) and Barakat et al. (1984) explain the nonspecific ability of *C. pseudotuberculosis ovis* to raise the resistance of sheep to artificial infection with potential pathogens in a manner comparable with that produced by BCG, as the waxy material in the cell wall structure of *C. pseudotuberculosis ovis* appears to stimulate antibody production in the same way that Mycobacteria waxes do.

CONCLUSION

It is concluded that the *C. pseudotuberculosis ovis* bacterin has nonspecific immune-stimulating effects against *Pasteurella* species in sheep vaccinated with inactivated Pneumobac® and produces a better immune response. Moreover, further studies on the nonspecific immune stimulating effect of inactivated *C. pseudotuberculosis ovis* on other bacterial and viral species are recommended to improve vaccine potency. Also, more immunological studies on the *C. pseudotuberculosis* bacterin and its toxin are required to determine how they work to enhance immunity against other bacteria. Finally, the obtained results are essential for developing the *Pasteurella* vaccine.

DECLARATIONS

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Authors' contribution

In the present study, all authors contributed to this research work. Eman Mohamed EL-Rawy did an experimental design. Wafaa Sayed Ahmed and Marwah Mohamed Mohamed prepared the vaccines. Marwa Magdy Sayed Khedr and Abeer Abdelsadek Ahmed Mwafy collected the blood samples and did the statistical analysis. Wafaa Sayed Ahmed,

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Abeer Abdelsadek Ahmed Mwafy, Marwah Mohamed Mohamed, and Marwa Magdy Sayed Khedr did the evaluation of the immune response. All authors contributed equally to the writing and review of the manuscript, as well as to the collection of papers related to the research subject. The final version of the manuscript to be published in the present journal was read and approved by all authors.

Competing interests

The authors declare that there is no conflict of interest.

Ethical considerations

Ethical concerns, such as redundancy, misconduct, publishing consent, the fabrication or falsification of data, multiple submission or publication, and plagiarism have been verified by the authors.

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Effect of the Sublethal Dose of Lead Acetate on Malondialdehyde, Dopamine, and Neuroglobin Concentrations in Rats

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ABSTRACT

Lead can have detrimental behavioral, biochemical, and physiological effects on the body. The current experiment was designed to estimate the sublethal dose of lead acetate that induce oxidative stress on the central nervous system (CNS) in adult using the probit analysis. Moreover, the current study examined the dose-response curve by successive doses of lead acetate on some parameters related to oxidative stress for 28 days. A total of 36 adult male rats were randomly selected and divided equally into six experimental groups and treated for 28 days. Rats in the control group received distilled sterile water, and those in G1, G2, G3, G4, and G5 were gavaged with 4, 8, 16, 32, and 64 mg/kg of lead acetate, respectively. The result indicated a positive correlation between the successive doses of lead acetate. Malondialdehyde concentration decreased dopamine and neuroglobin by increasing the dose of lead acetate in experimental groups (G3, G4, and G5), compared to the control group. In conclusion, exposure to the sublethal dose of 16 mg/kg of lead acetate significantly alters the levels of the neurotransmitters and increases the production of oxidative stress in the CNS tissue.

Keywords: Central nervous system, Dopamine and Neuroglobin, Lead acetate, Malondialdehyde, Rat

INTRODUCTION

Lead poisoning is a recognized significant concern to the public's health, particularly in developing nations. Softness, high malleability, ductility, low melting point, and corrosion resistance qualities of lead have make it useful in different fields, including the automotive, paint, ceramic, and plastic sectors. Lead may be found in the water, paints, soil, and brass plumbing fittings (La-Llave-León et al., 2016). Tetraethyl lead and lead acetate are two inorganic and organic forms of the metal lead, respectively, that can be found in nature (Shalan et al., 2005; ATSDR, 2017). Increased production of reactive oxygen species (ROS), which interfere with the antioxidant formation, is the main cause of lead poisoning. Lead produces ROS such as singlet oxygen, hydrogen peroxide, and hydroperoxide (Klimkowicz-Pawlas et al., 2017).

Lead is a pervasive environmental neurotoxin that has harmful effects on the host's behavior and neurochemistry and causes neuronal abnormalities even in trace amounts of exposure. The developing central nervous system (CNS) is vulnerable to Pb neurotoxicity, and any Pb exposure throughout CNS development can have long-lasting negative effects on the CNS's pathway of development as well as neuronal signaling and plasticity, leading to modifications in cognitive and behavioral characteristics that last well into adulthood. The Pb-induced neural signaling disturbance has been linked to a number of molecular processes, including increased oxidative stress, changes in the biochemistry of neurotransmitters, and mitochondrial dysfunction. However, these Pb-mediated changes in synapse growth and function are still poorly understood (Ahmad et al., 2018; Imosemi et al., 2020). The nervous system seems to be the most vulnerable and prime target for Pb-induced poisoning when compared to other organ systems, as it can result in death or lasting CNS damage at higher doses (Imosemi et al., 2020).

Oxidative stress is reportedly one of the mechanisms generating the CNS neurotoxicity caused by Pb exposure (Salim, 2017; Imosemi et al., 2020). Several studies have shown increased lipid peroxidation, reduced glutathione, and superoxide dismutase (SOD) activity in the CNS homogenates of Pb-treated rats (El-Masry et al., 2011; Fu and Xi, 2020). The hippocampus plays a crucial role in animal memory, learning, and spatial cognition (Alexandrov et al., 2013; Wani and Usmani., 2015). The cerebellum also controls muscle tone, saccadic and smooth eye movements, balance, and motor coordination (Pal et al., 2015; Wolf and Lappe, 2021). The architecture, shape, and function of the afflicted portions will be impacted by the Pb poisoning of the affected brain elements. High production of reactive oxygen species (ROS) and disruption of antioxidant production are the main causes of Pb poisoning. In the body, glutathione controls

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ROS. Lead also has an impact on other antioxidant enzymes, including catalase and superoxide dismutase (Navabpour et al., 2021). Lipid peroxidation caused by an increase in oxidative stress damage cell membranes (Al-Okaily and Al-Shammari, 2017; Klimkowicz-Pawlas et al., 2017). Therefore, the current study was designed to evaluate the sublethal dose of Pb acetate that induced oxidative stress in CNS by measuring some criteria related to CNS.

MATERIALS AND METHODS

Study

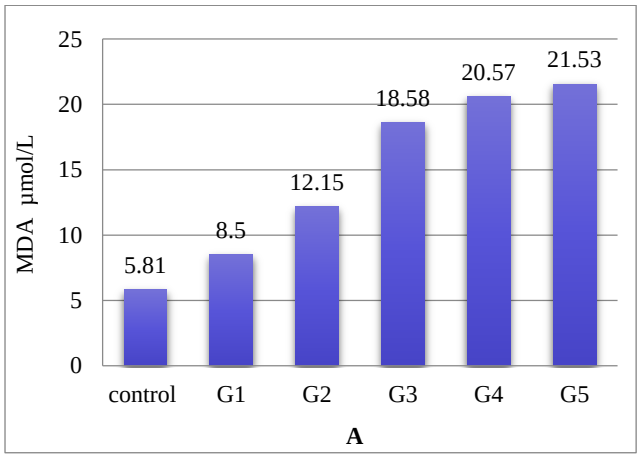
A total of 42 adult male rats weighing between 190 and 220 grams were used in the current study. Animals were housed at 22-25 °C with a 12-hour light/dark cycle. Throughout the testing period, animals had unrestricted access to water and pellets. After acclimatization for 15 days, rats were randomly selected and divided equally into six experimental groups and treated for 28 days. Rats in the control group received sterile distilled water. Those in the experimental groups G1, G2, G3, G4, and G5 received 4, 8, 16, 32, and 64 mg/kg/ orally/ day of lead acetate for 28 days, which is equal to 1/1120,1/560,1/280, 1/140, 1/70 from LD50 of Pb acetate respectively (Ibrahim et al., 2011). Blood samples were taken after the therapy, and the following factors were measured at 28 days of the experiment. Malondialdehyde (MDA) concentration (µmol/l) was determined by a modified procedure as described by Guidet and Shah (1989), and the neuroglobin and dopamine concentrations (pg/mL) were measured using the commercially available ELISA Kit (CEA851Ge, Cloud-Clone Corp Com, USA) according to the manufacturer's instructions.

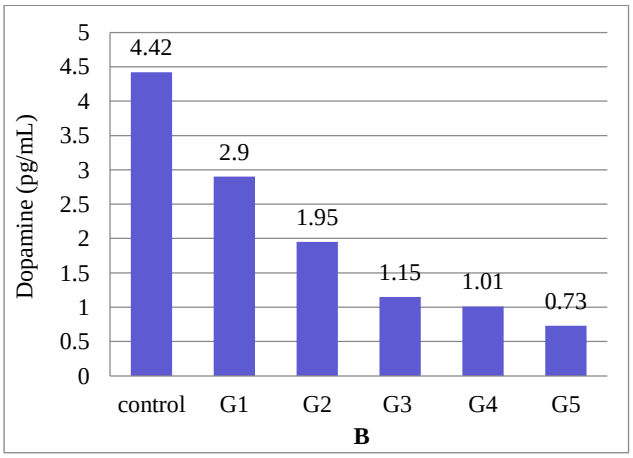
Statistical analysis

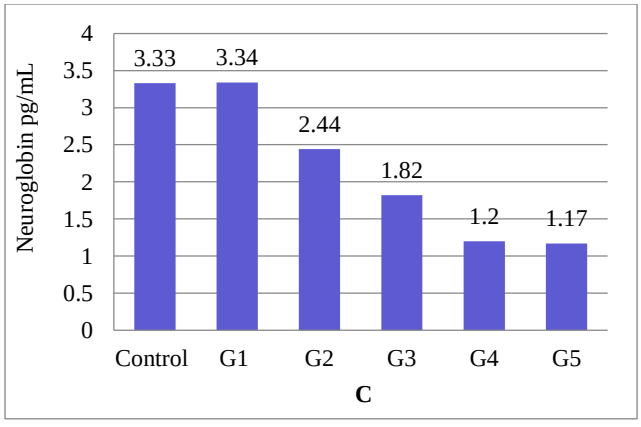
The collected data were analyzed in SPSS (Version 22) by using One-Way Analysis of Variance (ANOVA), LSD test was selected to find the significant level between the different data at the level of $p < 0.05$ (Snedecor and Cochran 1980).

RESULTS AND DISCUSSION

As can be seen in Graph 1, there was a significant ($p<0.05$) elevation in MDA concentration (Graph 1-A) and a significant ($p<0.05$) decline in dopamine (Graph 1-B) and neuroglobin (Graph 1-C) concentration in relation to repeated Pb acetate doses as compared to the control group. Additionally, there was a non-significant ($p>0.05$) difference between the G4 and G5 groups in terms of neuroglobin and MDA concentration (Graph 1-A and Graph 1-C). Additionally, the control, G1, and G2 groups did not differ significantly regarding blood dopamine levels (Graph 1-B, $p>0.05$).







Graph 1. Comparison of administering different doses of lead acetate on serum MDA (**A**), dopamine (**B**), and Neuroglobin concentrations (**C**) after 28 days in male rats (n = 6). Values are expressed as mean ± standard error.

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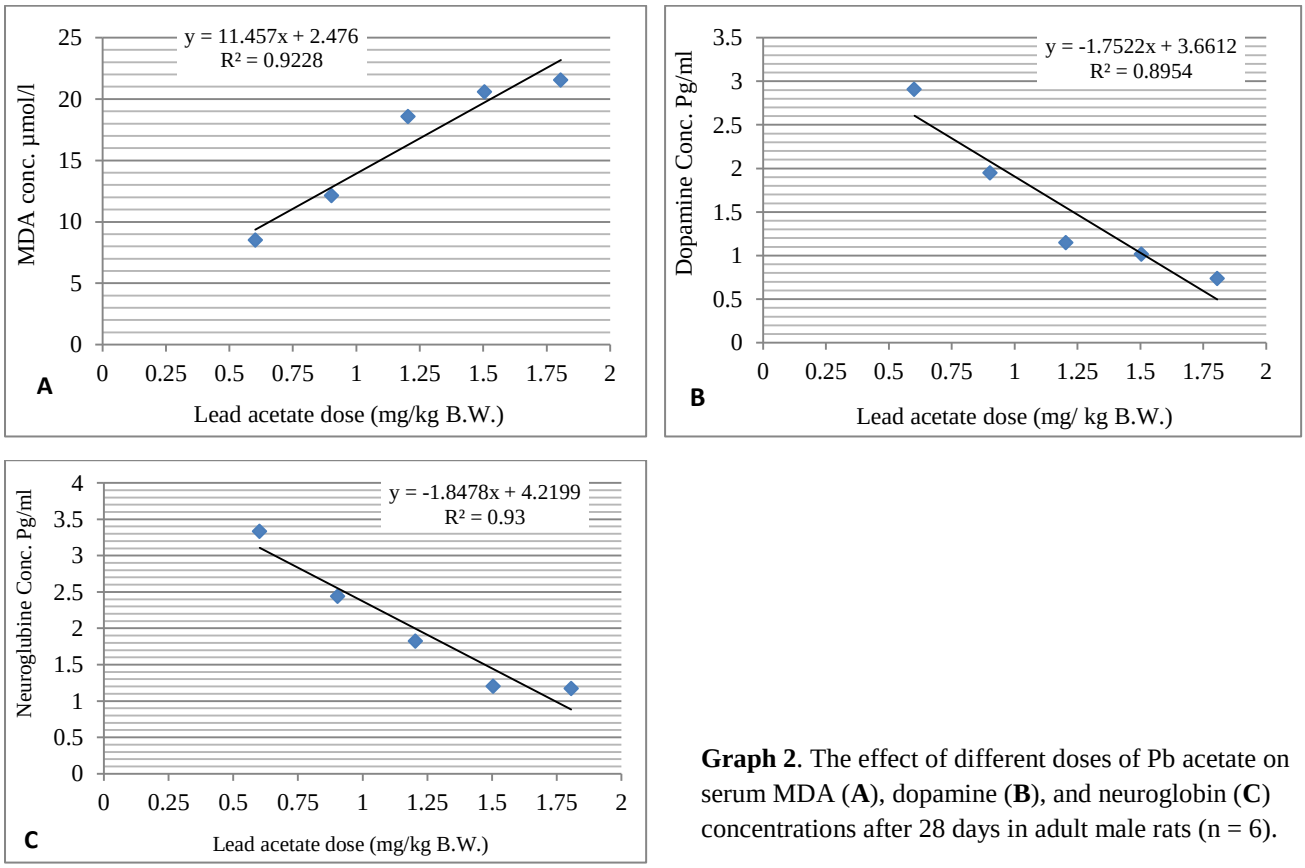
Determination of the sublethal dose of lead acetate

The obtained results of Graph (2-A) explained a highly significant ($p < 0.05$) increase in serum MDA concentration accompanied by a successive increase in the dose of Pb, while a significant negative relationship was observed between serum dopamine (2-B) and neuroglobin (2-C) concentrations ($p < 0.05$). According to probit analysis, the estimated sublethal dose of Pb was equal to 16 mg/Kg BW (1/280 mg/kg BW from LD50 of Pb).



Graph 2. The effect of different doses of Pb acetate on serum MDA (**A**), dopamine (**B**), and neuroglobin (**C**) concentrations after 28 days in adult male rats (n = 6).

DISCUSSION

The dose-response curve was employed using consecutive doses of Pb acetate to estimate the sub-lethal dosage of Pb acetate. The findings revealed a highly significant increase in serum MDA levels together with a drop in dopamine and neuroglobin levels. The current results are in agreement with those reported by Abdel-Wahab and Metwally (2014), Velaga et al. (2014), and Sutaria et al. (2022). Lead-induced neurotoxicity has been linked to oxidative stress, caused by the disruption of the antioxidant balance in cells (Jafarzadeh et al., 2022), dysregulation of cell signaling, and altered neurotransmission (Li et al., 2016).

The findings indicated a significant reduction in dopamine and neuroglobin concentrations with increasing doses of Pb acetate. The Pb exposure to cells or tissues might harm the nervous system through various pathways. It could damage glial cells, mainly in the cerebral cortex, cerebellum, and hippocampus, pass the blood-CNS barrier, and interfere with the structural elements of the CNS (Gandhi and Abramov 2012). As reported, it is preferentially deposited in specific CNS areas and is linked to aberrant behavior, learning disabilities, diminished hearing, neuromuscular weakness, and poor cognitive capabilities in experiment people and animals (Verina et al., 2007). A wide range of neurological illnesses, including mental retardation, behavioral issues, nerve damage, Alzheimer's disease, schizophrenia, and Parkinson's disease, might even be brought on by it, in addition to other biochemical abnormalities (Jaya Prasanthi et al., 2005; Bazrgar et al., 2015).

Lead can prevent the release of neurotransmitters from the preganglionic neurons since Pb is known to play a role in the synthesis and release of neurotransmitters. The Pb neurotoxicity can cause changes in cholinergic and dopaminergic neurotransmission in the CNS, including serotonin, dopamine, norepinephrine, and acetylcholinesterase activity, which are the first behavioral disorders in people and animal models (Flora et al., 2012; Malavika et al., 2021). Lead is thought to have a role in the transmission of processes in dopaminergic, cholinergic, catecholaminergic, and serotonergic neurons. In animal models, it has been shown that Pb interacts with a number of neurotransmitter systems, such as the dopamine, norepinephrine, serotonin, and gamma-aminobutyric acid systems (Mao et al., 2013; Chen et al., 2021). Lead can therefore prevent the utilization of neurotransmitters without selectivity in neuronal conductivity (Ferizi et al., 2020).

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CONCLUSION

The obtained results of the current study reveal that exposure to Pb acetate can significantly affect neurotransmitter levels, increase oxidative stress formation, and harm neurological functions. The sublethal dose of Pb was determined by calculating the concentrations of some indicators (MDA, dopamine, and neuroglobin). Therefore, future studies can be conducted by analyzing factors, such as histological changes or other neurotransmitters and animal behaviors.

DECLARATIONS

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Authors' contribution

All authors checked and approved the final version of the manuscript.

Competing interests

There is no conflict of interest.

Ethical consideration

All authors have reviewed the manuscripts for ethical concerns, such as plagiarism, permission to publish, misconduct, data fabrication and/or falsification, multiple publishing and/or submission, and redundancy.

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The Expression of Plasma Protein in Bali-polled Bulls Using 1D-SDS-PAGE

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ABSTRACT

The fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. Factors, such as the interstice factor of the sperm and the plasma component of semen, affect the fertility rate of bulls. The fertility rate can also be determined by identifying the protein content of semen plasma. Therefore, the current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bulls. The study was conducted at the Laboratory of Semen Processing, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia, the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia from November 2021 to January 2022. The samples came from 5 Bali-polled and 5 Bali-horned bulls. Semen collection was conducted twice a week using an artificial vagina. The concentration of seminal plasma protein was determined by the Bradford method of 1D-SDS-PAGE. The study results showed that fresh semen of Bali-polled and Bali-horned bulls was considered a normal category. Seminal plasma proteins of Bali-polled and Bali-horned bulls were classified using 8 bands to categorize molecular weight; 150 kD (IGF-1), 110 kD (A-kinase anchoring protein 3), 93 kD (A-kinase anchoring protein 4), 54-87 kD (Arylsulfatase-a), 44-62 kD (N-Acetyl-ß-Guicosaminidase), 44kD (Phosphoglycerate kinase), 15-30 kD (BSP A1/A2, BSP-A3 and BSP-30 [BSP1, BSP3, and BSP5]) and 12-14 kD (Acidic seminal fluid proteins). The findings indicated that both Bali-polled and Bali-horned bulls could have a high reproductive rate. In conclusion, protein analysis based on molecular weight using 1D-SDS-PAGE can be used as a biomarker for semen quality in Bali-polled bulls. Therefore, evaluating the semen quality with a molecular basis as an additional indicator of superior bull in the selection process is an alternative method.

Keywords: Bali-polled bull, Seminal protein plasma, Sperm, 1D-SDS-PAGE

INTRODUCTION

Livestock reproductive biotechnology has now developed and opened up great opportunities to explore the potential for reproductive performance, as well as increasing population, and genetic quality of livestock. Artificial insemination (AI) is the first generation of reproductive technology that aims to efficiently utilize superior males, avoid the spread of reproductive diseases, and improve the genetic quality of livestock (Syahruddin, 2020). Artificial insemination technology has the potential to be used for the development of Bali-polled cattle in Indonesia. Thus, the selection of polled bulls becomes very important, especially in modern livestock management (Brockmann, 2000).

Conventionally, the breeding soundness evaluation (BSE) technique was used to test the reproductive ability of an animal. The BSE is repeatable, easy to perform, and correlates with male fertility (Thundathil et al., 2016). The assessment method of BSE is not limited to the bulls that can reach the BSE threshold or exceed it. The method provides an opportunity for males with low potential to be selected in case only one aspect of the BSE test is observed (Alexander, 2008).

According to Kaya and Memili (2016), the fertility rate of bulls in a breeding program is not only described by the quantity and quality of semen. In the same line, Viana et al. (2018) found that many factors contributed to determining the quality of sperm, such as the interstice factor of the sperm and the plasma component of semen. Moreover, the protein function affects the fertility rates in bulls depending on the protein content of semen plasma (Druart and de Graaf, 2018).

The protein in seminal plasma plays an important role in sperm protector regulations (Fu et al., 2019). Understanding the physiological way seminal plasma proteins' function can help breeder bulls become more fertile and have less infertility. Some specific proteins in semen have been linked to fertility although many seminal proteins still have unresolved functions and correlations with fertility indices (Kumar et al., 2012). Therefore, there is a need for

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further studies to investigate the relationship between semen plasma protein and bulls' fertility. The current study aimed to identify the relationship between seminal plasma protein molecular weight and semen quality of Bali-polled bull.

MATERIALS AND METHODS

Ethical approval

All procedures in the present study were approved by the University Animal Ethics Committee of Hasanuddin University, Makassar, Indonesia.

Study area and period

The study was conducted at the Laboratory of Animal Reproduction, Semen Processing Unit, Faculty of Animal Science, Hasanuddin University, Makassar, Indonesia. Determination of seminal plasma protein was analyzed at the Research Center for Applied Zoology, National Research and Innovation Agency, Cibinong, Indonesia, and the Laboratory of Animal Biotechnology Center, IPB University, Bogor, Indonesia, from November 2021 to January 2022. The seminal plasma samples were obtained from 5 Bali-polled bulls and 5 Bali-horned bulls aged 5-8 years. The sample size was based on the previous studies by Kasimanickam et al. (2019) and Westfalewicz et al. (2021).

Semen collection and evaluation

Bali-polled and Bali-horned bull semen samples were collected using an artificial vagina twice a week. Soon after collection, the semen samples were sent to the laboratory for evaluation. The evaluation of semen quality was performed both macroscopically and microscopically. Semen that passed the macroscopical evaluation was then evaluated for microscopic evaluation, including motility, concentration, abnormality, viability, and membrane integrity of the sperms.

Motility

The motility, progressive motility, and kinematics of sperms were determined by making 10 µl semen spot on the object glass. For the movement evaluation of sperms, the semen was then subjected to the CASA (Vision Version^{TM} 3.7.5 program Minitube, Germany) following Diansyah et al. (2022).

Concentration

The sperm concentration was evaluated using a photometer SDM 6 (Minitube, Germany). The cuvette containing 3 ml of physiological NaCl solution was inserted into the device with the line facing forward, and then the zero button was pressed. The cuvette was removed and then replaced with a cuvette containing a physiological NaCl solution in which 30 µl of fresh semen was added, and then the result button was pressed; the concentration of spermatozoa would be obtained in the amount per ml (Diansyah et al., 2022).

Abnormality and viability

The viability and abnormality of the sperms were evaluated by mixing 10 µl of semen and 10 µl of Eosin 2% in the object glass. After drying, the object glass was observed using a trinocular microscope (Primo Star, Zeiss, Germany) at 400x magnification with Indomicro View 3.7 software. Spermatozoa with red color were considered dead, and the colorless ones were considered alive. Spermatozoa with severed tails, broken tails, and abnormal head shapes were considered abnormal. For the accurate calculation, at least 200 sperms cells per observation were performed following Diansyah et al. (2022).

Membrane integrity

An evaluation of membrane integrity was performed microscopically in which 10 µl of semen was added into HOST solution (0.179g NaCl in 100 ml of aquabides). The solution was then incubated for 30 minutes at 37^{o}C in the oven. The evaluation was carried out using a 400x magnification of a trinocular microscope (Primo Star, Zeiss, Germany) by counting at least 200 spermatozoa cells. Sperms with membrane integrity were characterized by a circular tail, while sperms characterized by a straight tail were considered damaged (Diansyah et al., 2022).

Concentration of seminal plasma protein

Seminal plasma protein concentration was determined by centrifuging the semen at about 3-4 mL at 6500 rpm for 30 minutes. After centrifugation, the supernatant was put into the microtube and kept in a cryobox for storage at 20^{o}C. For characterization of seminal plasma protein, 1D-SDS-PAGE (SMOBIO, Hsinchu, Taiwan) based on molecular weight (MW) of protein was used. The gels were stained (Sigma-Aldrich®, United States) with Coomassie Brilliant Blue stain (Sigma-Aldrich®, United States), and molecular mass was determined by the MW marker (Karunakaran et al., 2019). Concentration of seminal plasma protein was determined by the Bradford method (Bradford, 1976). The

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Bradford protocol of analysis using Protein Assay Kit (Sigma-Aldrich®, United States) was based on the user guide of coomassie (Bradford, 1976) for sample preparation. The samples were then analyzed by Thermo Skanlt RE for Multiskan Go Software, 3.2 version (Thermo Scientific®, United States) to obtain seminal plasma concentration.

Regarding the separation of seminal plasma protein, two polyacrylamide gels 12% (containing sodium dodecyl sulphate [SDS]) were used. A 20 µg of protein mass was analyzed with SDS-PAGE using Thermo Prestained Protein Ladder marker (5 µL, Thermo Scientific®, United States). Subsequently, the protein separation was performed using 120 v electricity in 70 minutes. The photo of gel color was dipped in an acidic liquid and then shaken until protein bands appeared to ease for identifying protein bands. Each protein band was determined using ImageJ software (Schneider et al., 2012).

Statistical analyzes

The data of fresh semen quality both in Bali-polled and Bali-horned bulls were reported as mean and standard deviation. T-Test was used to compare each parameter regarding the quality of fresh semen in the two bulls. The parameter was considered significant when the p-value was lower than 0.05. All statistical analyzes were calculated using SPSS for Windows software (version 25). The specific protein in each seminal plasma of the bulls was analyzed descriptively.

RESULTS AND DISCUSSION

The quality of fresh semen and concentration of seminal plasma protein in Bali-polled and Bali-horned bulls

This current study intended to explore the quality of Bali-polled bull semen for predicting the fertility level using plasma protein expression. In order to achieve this intention, Bali-horned bulls that have been known as having high fertility level was used for comparison. Table 1 shows the quality of Bali-polled and Bali-horned bulls' fresh semen. Statistical analysis showed that sperm motility in the Bali-polled bulls did not differ significantly ($p > 0.05$) from the Bali-horned bulls (82.91% vs. 83.18%). Likewise, sperm concentration (1578.72 x 10^6 mL^{-1} vs. 1475.80 x 10^6 $mL^{-1)}$, sperm viability (91.97% vs. 92.03%), sperm intact membrane (80.90% vs. 81.52%), and seminal plasma concentration (567 mg mL^{-1} vs. 547 mg mL^{-1}) did not differ significantly between the two groups ($p > 0.05$). However, sperm abnormality in Bali-polled bulls was significantly ($p < 0.05$) lower than in Bali-horned bulls (4.17% vs. 5.19%). The results of the current study revealed that the quality of fresh semen in both groups of bulls was likely higher than the other local bulls reported previously (Romadhoni et al., 2014; Zulyazaini et al., 2016).

According to Indonesian Minister of Agricultural Regulation Number:10/Permentan/PK.210/3/2016 and Indonesian National Standardization 4868.1:2007 for frozen bull semen (Baharun et al., 2021), fresh semen quality can be processed as frozen semen with sperm motility value >70% and sperm abnormalities value <20%. Based on these regulations, the quality of Bali-polled and Bali-horned bulls' fresh semen in the present study was considered a normal category. Therefore, the semen of the two bulls used in this study can be processed further as frozen semen.

As can be seen in Table 1, the seminal plasma protein concentration of Bali-polled and Bali-horned bulls was 567 mg mL^{-1} and 547 mg mL^{-1}. The plasma protein concentration was used only as the basis for further electrophoresis or mass spectrometry analysis by Bradford method. The amount of plasma protein concentration cannot be relied upon to analyze the quality of semen (Westfalewicz et al., 2016). However, several studies have demonstrated that plasma protein components in semen are considered effective in improving sperm quality (Codognoto et al., 2018; Viana et al., 2018; Panda et al., 2020). Seminal plasma is mostly composed of testicular, epididymal, and accessory sex gland secretions. Proteins involved in sperm metabolism and motility, membrane restructuring and function, protection against reactive oxygen species and immunological responses, capacitation, and the acrosome reaction are expressed in the fluid surrounding sperm cells in semen (Moura et al., 2018). Many proteins in the seminal plasma bind the sperm, affecting membrane structure and sperm function. The study of Purdy (2006) has shown that the role of seminal plasma proteins in regulating sperm function is highly complex. Several studies have provided solid evidence that seminal plasma proteins were adsorbed to the sperm surface and affected its function and properties (Purdy, 2006; Moura et al., 2018).

Table 1. The quality of fresh semen and seminal plasma protein concentration of Bali-polled and Bali-horned Bulls

Parameters / Breed	Sperm motility (%)	Sperm concentration (10^6 mL^{-1})	Sperm viability (%)	Sperm abnormality (%)	Sperm intact membrane (%)	Seminal plasma protein concentration (mg mL^{-1})
Bali-Polled	82.91 ± 1.34	1578.72 ± 65.83	91.97 ± 10.6	4.17 ± 2.39[a]	80.90 ± 1.04	567
Bali-Horned	83.18 ± 1.44	1475.80 ± 59.93	92.03 ± 6.96	5.19 ± 1.45[b]	81.52 ± 0.71	547
p value	0.944	0.219	0.943	0.014	0.313	

Means in a column with different superscripts differ significantly at $p < 0.05$.

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The specific protein profile of seminal plasma in Bali-polled and Bali-horned bulls

The specific profile of the targeted protein in this study affected the quality of spermatozoa, as indicated by the molecular weight of the protein using 1D-SDS-PAGE (Figure 1). The seminal plasma protein-specific profile of Bali-polled and Bali-horned bulls, as determined by 1D-SDS-PAGE, shared the same particular protein from 8 protein candidates. The specific profile of the targeted protein is shown in Table 2. Selvaraju et al. (2016) indicated protein with MW 150 kD represented IGF-1. This protein improves sperm motility by reducing oxidative stress, maintaining structural membrane integrity and mitochondrial membrane potential, and protecting calmodulin, dermcidin, and the sperm acrosome membrane (Selvaraju et al., 2016). IGF-1 is found in plasma and tissue fluid, influencing steroidogenesis, metabolism, cell proliferation, and differentiation (Colombo and Naz, 1999). IGF-1 also affects various reproductive processes and plays an essential role in the onset, progress, and regulation of spermatogenesis (Dyck et al., 1999).

Table 2. Seminal plasma protein-specific profile of Bali-polled and Bali-horned bulls assessed by 1D-SDS-PAGE

Protein	MW (kD)	Bali-Polled	Bali-Horned
IGF-1	150	+	+
A-kinase anchoring protein 3	110	+	+
A-kinase anchoring protein 4	93	+	+
Arylsulfatase-a	54-87	+	+
N-Acetyl-ß-Guicosaminidase	44-62	+	+
Phosphoglycerate kinase	44	+	+
BSP A1/A2, BSP-A3 and BSP-30 (BSP1, BSP3 and BSP5)	15-30	+	+
Acidic seminal fluid proteins	12-14	+	+
Total Protein		8/8	8/8

MW: Molecular weight, +: Protein expressed, -: Protein non-expressed, BSP: Binder sperm protein

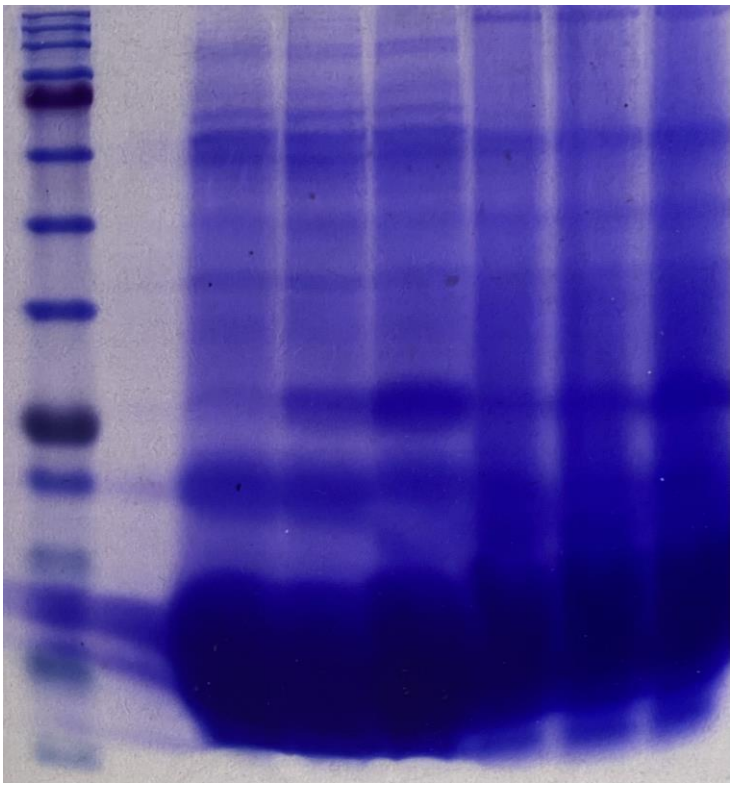

Figure 1. 1D-SDS-PAGE of Bali-polled and Bali-horned bulls

The protein with MW 110 kD and 93 kD contains AKAP 3 and AKAP 4 (Pujianto et al., 2018). These proteins contain the fibrous coating of the sperm's outer dense fiber, which forms the axoneme (Carr and Newell, 2007). During capacitation, AKAP3 and AKAP4 are phosphorylated, which is crucial for maintaining sperm motility (Pujianto et al., 2018). Molecular function mediates sperm motility through A-Kinase Anchoring Protein 4 (AKAP4) setting. It correlates with Adenosine triphosphate (ATP) for sperm motility and ATP's dephosphorylation (Freitas et al., 2017). AKAP3 is degraded in bovine sperm incubated under capacitation conditions (Hillman et al., 2013). The AKAP3 and AKAP4 isoforms are uniquely expressed by spermatids and spermatozoa, localize in the flagellum, and are involved in sperm motility. AKAP3 is mainly localized at the principal piece of the tail (Lea et al., 2004).

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The MW 54-87 kD protein may represent arylsulfatase-a (Moura et al., 2010). This protein can be attached to the superficial sperm head (Weerachatyanukul et al., 2003). During the binding/penetration process, arylsulfatase-a functions as either lectins or hydrolases (Nickolajczyk and O'Rand, 1992). Arylsulfatase-a is involved in the stability and permeability of the spermatozoa plasma membrane (Gadella et al., 1991). Arylsulfatase-a quantitative detection and distribution during in vitro sperm capacitation could be used to get a better insight into molecular changes during the fertilization process and improve artificial reproductive technologies (Gómez-Torres et al., 2021). The protein N-acetyl-β-guicosaminidase (62-44 kD) is glucose hydrolyze enzyme for glycoprotein membrane during sperm maturation in the epididymis (Moura et al., 2010). These proteins are potential mediated sperm-oocyte interactions (Abascal et al., 1998).

The protein with MW 15-30 kD (Druart et al., 2013) is indicated as binder sperm protein (BSP) A1/A2, BSP-A3, and BSP-30 (BSP1, BSP3, and BSP5). After ejaculation, BSP proteins bind to the sperm membrane at the acrosome, post-acrosome, and midpiece area, which is crucial for beginning motility (Manjunath et al., 1994). All biochemical studies and binding properties have provided good insight into the putative functions of BSP proteins in fertility. The BSP proteins are multifunctional proteins used for various purposes, including sperm motility, formation of the sperm reservoir, but most importantly, sperm capacitation (Plante and Munjunath, 2014). The function of BSP-30 kD as exhibits a significantly broader binding specificity to choline phospholipids, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, and cardiolipin, whereas BSP-A1, BSP-A2, and BSP-A3 bind specifically to the phosphorylcholine groups (Desnoyers and Manjunath, 1992).

According to Moura et al. (2010), a protein with MW 12-14 is Acidic Seminal Fluid Proteins (aSFP). Jobim et al. (2004) suggested the aSFP as a marker of good semen freezability. Furthermore, the aSFP is a marker for bovine semen freezability, possibly due to its antioxidant activity and effect on sperm mitochondrial function (Moura et al., 2010). In bulls, aSFP represents a major sex-specific seminal protein secreted mainly from the ampulla and seminal vesicle in variable but high concentrations (Einspanier et al., 1994). This protein seems to protect spermatozoa from free oxygen radicals in such an in vitro system (Schoneck et al., 1995).

Regarding the profile of seminal protein expression, Bali-polled and Bali-horned bulls contain similar total protein candidates. This suggests that the two bulls are linked and have a similar reproductive rate.

CONCLUSION

Protein analysis using 1D-SDS-PAGE based on molecular weight can be used as a biomarker for semen quality in Bali-polled bulls. The semen of both bulls contains IGF-1, AKAP 3, AKAP 4, arylsulfatase-a, N-acetyl-β-guicosaminidase, BSP A1/A2, BSP-A3, and BSP-30 (BSP1, BSP3, and BSP5) and aSFP candidates that are linked to a high reproductive rate. Therefore, the evaluation of the semen quality with molecular basis as an additional indicator of superior bull in the selection process is one of the alternatives.

DECLARATIONS

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Authors' contribution

Athhar Manabi Diansyah contributed to collecting data, drafting method, analysis, writing-original draft, writing-review, and editing the manuscript. Muhammad Yusuf contributed to performing data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft and approved the final manuscript. Abdul Latief Toleng contributed to performing data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft and approved the final manuscript. Muhammad Ihsan Andi Dagong performed data analysis, writing-original draft, reading-original draft, writing-review, and reading-review draft, and approved the final manuscript. Tulus Maulana performed data analysis, writing-original draft, reading-original draft, writing-review, reading-review draft, and approved the final manuscript.

Competing interests

The authors declared no competing interests.

Ethical consideration

The authors have confirmed ethical issues, such as plagiarism, misconduct, information fabrication and/or falsification, consent to publish, duplicate publishing and/or submission, and redundancy.

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Amelioration of Hepatotoxicity by Sodium Butyrate Administration in Rats

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ABSTRACT

Lead poisoning is a serious environmental issue with life-threatening consequences. Lead poisoning increases the risk of cancers, gastrointestinal disorders, hepatotoxicity, central nervous system diseases, nephropathy, and cardiovascular diseases in animals and humans. The current study aimed to investigate the effect of sodium butyrate, as an antioxidant, on protecting female adult rats from the harmful effects of lead acetate. A total of 40 adult female albino rats were divided randomly into four equal groups. The first group dealt as the control. The second group received lead acetate at a dose of 200 mg/kg daily orally. The third group received lead acetate at a dose of 50 mg/kg daily orally, and the fourth group received both sodium butyrate and lead acetate orally/day for 35 days. The result indicated that sodium butyrate reduced the concentration of liver enzymes (ALT, AST, and ALP) which were elevated by lead acetate poising. Moreover, sodium butyrate ameliorates the redux status by decreasing malondialdehyde and increasing total antioxidant capacity. Additionally, sodium butyrate-treated rats showed significant alterations in the expression of peroxisome proliferator-activated receptor gamma and interleukin -10 genes. In conclusion, this study reveals an unrecognized role for peroxisome proliferator-activated receptor gamma and Interleukin-10 signaling after sodium butyrate treatment in regulating the immunopathology that occurs during lead acetate poising.

Keywords: Interleukin-10, Lead acetate toxicity, Sodium butyrate, PPAR-gamma, Rat

INTRODUCTION

Environmental pollutants from industry, such as lead, are widespread. The earth's crust contains a significant amount of pollutants, which can be easily dispersed in the environment. A major environmental disease with potentially fatal effects is lead poisoning. Lead poisoning has been linked to various cancers, gastrointestinal tissues, and cardiovascular diseases in humans and animals, as well as hepatotoxicity, nephrotoxicity, and changes to the central nervous system (Yousef et al., 2019).

Lead can cause oxidative stress, which can cause liver damage, by increasing the generation of free radicals to a certain level and reducing the effectiveness of the antitoxin system (Widiarko and Permata 2020). Butyric acid is a naturally occurring short-chain volatile fatty acid that is employed in the chemical, culinary, pharmaceutical, and animal feed sectors (Brändle et al., 2016). Research has concentrated on strain generation using metabolic engineering and process development employing inexpensive biomass feedstocks for the fermentation-based manufacture of bio-based butyric acid (Zigova and Šturdík, 2000). Butyric acid can potentially reduce harmful intestinal microorganisms while increasing digestibility coefficients. As a result of an increase in the small intestine's absorptive surface and the formation of beneficial bacteria in the gastrointestinal tract, sodium butyrate supplementation can boost feed intake and weight gain (Sikandar et al., 2017). In this regard, sodium butyrate has gained particular attention due to its capacity to suppress mucosal apoptosis via antioxidative, antibacterial, anti-inflammatory, and immunomodulatory effects (Zhang et al., 2015).

Peroxisome proliferator-activated receptor-gamma (PPAR-g) has pleiotropic effects on lipid metabolism, inflammation, and cell proliferation (Ferreira et al., 2014). Since PPAR-g ligands are known to have anti-inflammatory properties, both natural and synthetic versions of them have been tested in experimental models of sepsis. Treatment with Thiazolidinediones and natural PPAR-g ligands can decrease the release of interleukin-6 and interleukin-10 (IL-10), tumor necrotic factors-α, and Chemokine (C-C motif) ligand 2, as well as neutrophil infiltration in the liver and lung brought on by polymicrobial sepsis or endotoxemia (Chima et al., 2008; Han et al., 2017). The PPAR-g agonists also lessen high Mobility Group Box 1 (HMGB1) levels and indicators for organ failure in endotoxemic mice. As a result, the treatments dramatically boost the survival of mice (Collin et al., 2004; Kaplan et al., 2005; Lee et al., 2012).

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It is not yet clear how PPAR-g agonists reduce inflammation. According to a widely recognized theory, active PPAR-g directly interacts with a transcription factor and causes transrepression (Schmidt et al., 2010; Wu et al., 2020). Hemorrhagic shock-induced IkappaB kinase-related kinases activity and IkappaB kinase degradation are decreased by the synthetic PPAR-g agonist pioglitazone (Chima et al., 2008).

The pleiotropic cytokine IL-10, commonly referred to as cytokine synthesis inhibitory factor, plays a crucial role in immunoregulatory processes (Verma et al., 2016). It contains anti-inflammatory qualities and affects the function of several immune system cell types. The main sources of IL-10 secretion include activated T cells, monocytes, macrophages, dendritic cells, natural killer cells, and B cells (Blanco et al., 2008). Excessive IL-10 expression encourages the growth of some lymphomas and melanomas by stifling the antitumor immune response (Huang et al. 1999; Saraiva et al., 2020). The serum level of IL-10 may also signal illness progression. According to a study on individuals with radically resected solid tumors, IL-10 serum levels recover to normal; however, the IL-10 level was found to be continuously high in cases of tumor recurrence (de Vita et al. 2000). The survival and persistence of intracellular infections *in vivo* are fundamentally influenced by IL-10, such as *Leishmania donovani* (Chandra and Naik, 2008). When macrophages are inactivated by IL-10, fewer pro-inflammatory cytokines and reactive oxygen species that might otherwise cause cell damage are produced (Kessler et al., 2017). Therefore, the current study aimed to evaluate the effect of sodium butyrate on enhancing hepatotoxicity by PPAR signaling in rats.

MATERIALS AND METHODS

Ethical approval

All experiences were approved by the College of Veterinary Medicine Ethical Committee (COVM-6341).

Study design

The investigated samples in the current study were derived from 40 mature Wister Albino female rats aged 8 weeks with a mean weight of 190-200g. The rats were acclimatized in the animal home of the University of Baghdad, College of Veterinary Medicine, Baghdad, Iraq. The period of treatment was 35 days. During the trial, they were kept in well-ventilated rooms within plastic cages, fed a regular pellet diet, and given free access to water for one week. The indoor temperature was 20-25°C and 5% humidity. The rats were exposed to 12-hour alternating light/dark periods throughout the experiment. The bed was changed twice every week. The rats were randomly divided into four equal groups. The first group was considered a control (C), the rats in the second group were orally given sodium butyrate (SB) at a dose of 200 mg/kg daily (Alrafas et al., 2020), and those in the third group were orally subjected to lead acetate (LA) at a dose of 50 mg/kg daily (Sudjarwo et al., 2017). The rats in the fourth group were orally given SB+LA daily**.**

Blood collection and chemical analysis

Blood was collected from each rat using the eye technique (4 ml) because it is an easy technique that does not need any special instruction (van Herck 1998) and is one of the recommended methods of blood collection in rats. The rats in this method were anesthetized by intramuscular injection of Ketamine 60 mg/kg and Xylazine 12 mg/kg. The blood samples were centrifuged (GMBH, Germany) for 15 minutes at 3000 rpm to separate the serum, and the tubes were frozen at -20°C for subsequent analysis. Malondialdehyde (MDA) concentration (µmol/l) was determined by a modified procedure as described by Guidet and Shah, (1989), and the total antioxidant capacity were measured using the commercially available. Malondialdehyde concentration (µmol/l) was determined by a modified procedure as described by Guidet and Shah, (1989), and the total antioxidant capacity were measured using the commercially available ELISA kit (Biosource, USA) utilized according to the manufacturer's instructions. Alanine aminotransferase activity (ALT), alkaline phosphatase (ALP) activity, and aspartate aminotransferase activities (AST) concentrations are determined by a special kit (Spectrum AST, Egypt) using the device spectrophotometer (Sesil, England).

Dosage preparation

Sodium butyrate supplement capsules were purchased from Body Bio (USA). The SB administered dose to rats was 200 mg/kg (Alrafas et al., 2020), while LA obtained from the college laboratory was given at a dose of 50 mg/kg (Sudjarwo et al., 2017).

Quantitative Real-Time PCR

The performed Q-PCR to determine the expression of PPAR-g and IL-10) in the spleen (Table 1) was according to Mohammed et al. (2020). To this end, cDNAs were generated using total RNAs isolated from rats in control, SB, LA, and SB+LA groups using the Real MODTM Green W2 2x qPCR mix. Table 1 indicates the primers used for PPAR-g and IL10 (Lira et al., 2009).

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Table 1. Primer sequences of peroxisome proliferator-activated receptor gamma and interleukin -10

Primers	Reverse	Forward
PPAR-g	5′- GGTCCACAGAGCTGATTCCG -3	5′- GACTGAGTGTGACGACAAGATT -3
IL-10	5′- AGGCTTGGCAACCCAAGTAA -3′	5′- TCCGGGGTGACAATAACTGC -3′
References	5′- GCAGCGATATCGTCATCCAT -3′	5′- CCGCGAGTACAACCTTCTTG -3′,

PPAR-g: Peroxisome proliferator-activated receptor-gamma; IL-10: Interleukin -10

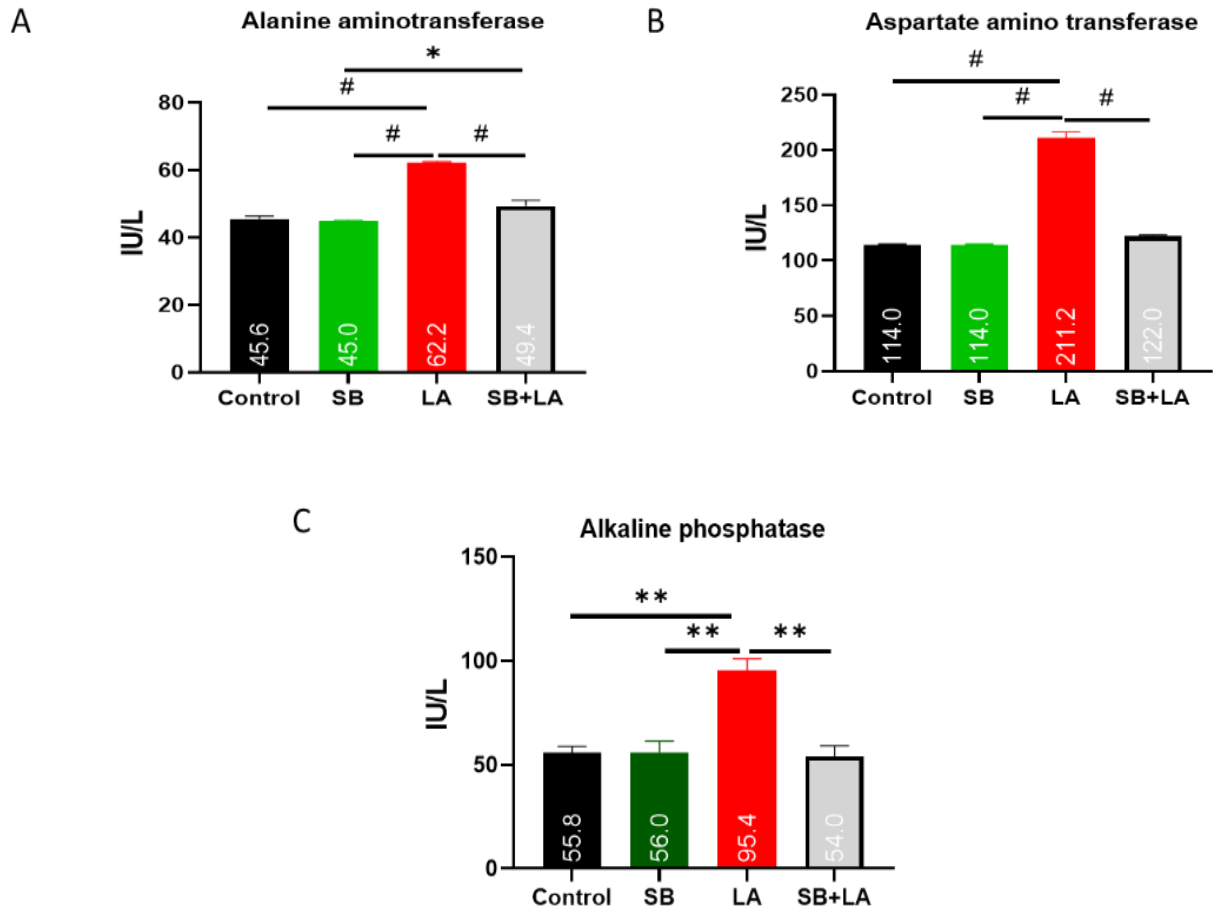
Statistical analysis

The collected data were analyzed in SPSS (Version 22) by using One-Way Analysis of Variance (ANOVA), LSD test was selected to find the significant level between the different data at the level of $p < 0.05$ (Snedecor and Cochran 1980).

RESULTS AND DISCUSSION

Sodium butyrate alleviates hepatotoxicity

As can be seen in Graph 1A, the serum ALT values in rats treated with LA significantly increased ALT activity, compared to the control, SB, and SB+LA groups ($p < 0.05$). In addition, ALT activity significantly decreased in the SB group compared with the LA group and SB+LA after 35 days ($p < 0.05$). Graph 1B shows the mean AST activity of the control and treated groups during the experiment. After 35 days of oral exposure to LA, a significant increase in AST activity was detected, the control, SB, and SB+LA groups ($p < 0.05$). In contrast, three groups of control, SB, and SB+LA indicated non-significant changes in terms of AST ($p > 0.05$). According to Graph 1C, ALP activity is significantly higher in LA group when compared to the control, SB, and SB+LA groups. The results also revealed that there were non-significant differences between the SB therapy and control groups at the end of the experiment ($p > 0.05$).



Graph 1. The effect of sodium butyrate, lead acetate, and their combination on liver function in adult female rats. * and **: Statistical significance between the groups at $p < 0.05$, $p < 0.01$, respectively. **A**: Serum alanine aminotransferase activity (IU/L). **B**: Serum aspartate aminotransferase activity (IU/L). **C**: Serum alkaline phosphatase activity (IU/L). Control: rats received only drinking water, SB: Rats orally received SB 200 mg/kg, LA: Rats orally received lead acetate 50 mg/kg, SB+LA: Rats orally received both sodium butyrate 200 mg\kg orally and LA 50 mg/kg.

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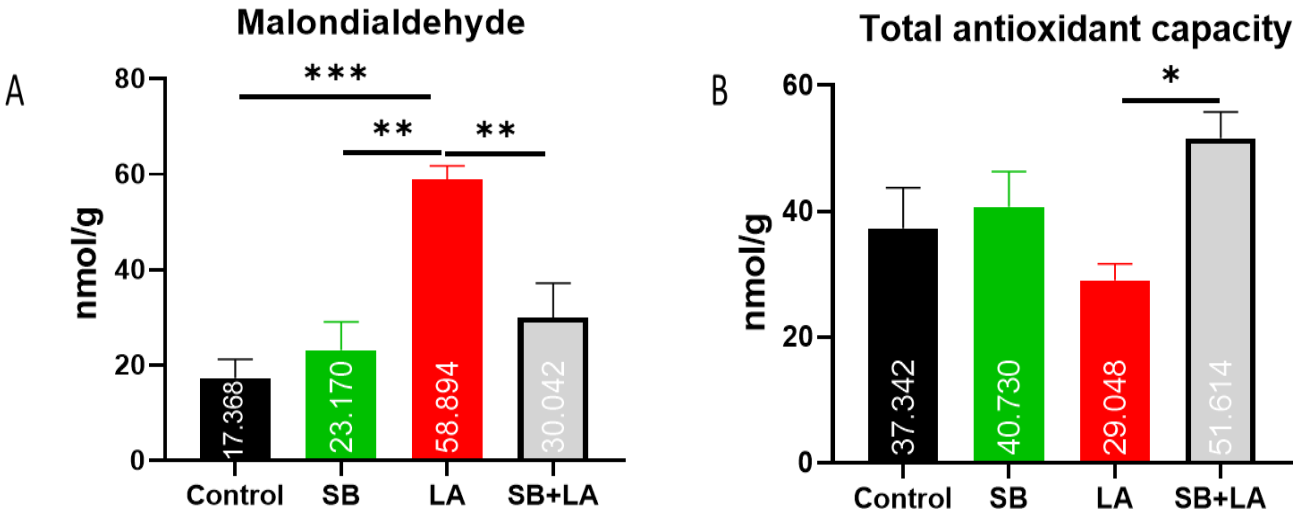
Sodium butyrate effects on the redux status

Graph 2A showed the mean MDA in adult rats' liver tissues after 35 days of treatment in the control and treated groups. Compared to the other experimental groups, there was a significant increase in MDA concentration in the liver of the LA group. Compared to the control group and group treated with only SB ($p < 0.05$).

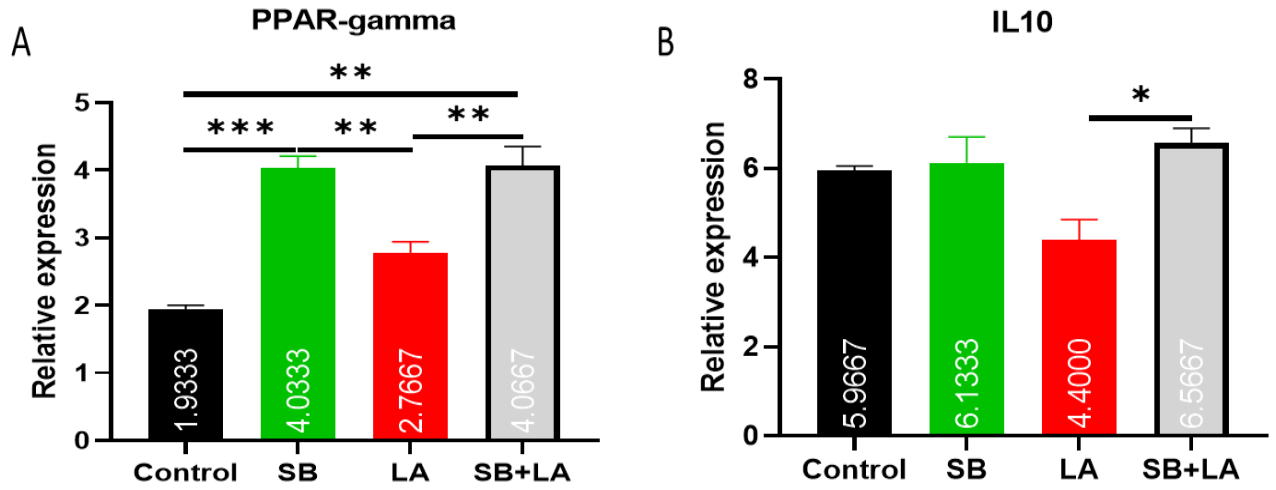
The values of serum total antioxidant capacity in rats exposed to LA and SB orally or SB+LA along the experimental period is clarified in Graph 2B. At 35 days, there were significant differences in the values of serum total antioxidant capacity in all experimental groups as compared to each other ($p < 0.05$). Treatment of rats with LA or SB+LA caused a significant increase in total antioxidant activity after 35 days of the experiment, compared to that of the control and SB groups ($p < 0.05$).



Graph 2. The effect of sodium butyrate, lead acetate, and their combination on redux status in adult female rats. *, **, and ***: Statistical significance between the groups at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. **A**: Malondialdehyde concentration in serum (nmol/g). **B**: Total antioxidant capacity concentration in serum(nmol). Control: Rats received only drinking water, SB: Rats orally received sodium butyrate 200mg/kg, LA: Rats orally received lead acetate 50 mg/kg, SB+LA: Rats orally received both sodium butyrate200 mg\kg and lead acetate 50mg/kg.

Validation of PPAR-gamma and IL-10 Genes in the spleen

The sodium butyrate group in Graph 3A showed a significant increase in PPAR-g, compared with a control group and group of rats gavaged with LA only ($p < 0.05$). PPAR-g expression was significantly decreased in the LA group compared with the SB+LAgroup ($p < 0.05$). Moreover, the (SB+LA) group was elevated significantly compared to the control group ($p < 0.05$). Graph 3B showed high elevation in the SB+LA group compared with the LA group ($p < 0.05$). Data showed a significant increase in PPAR-g and IL-10 after SB treatment ($p < 0.05$).



Graph 3. The effect of sodium butyrate, lead acetate, and their combination on PPAR-g and IL-10 in adult female rats. *, **, and ***: Statistical significance between the groups at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. **A:** An expression of PPAR- gamma gene. **B:** Expression of IL-10 gene. Control: Rats received only drinking water, SB: Rats orally received sodium butyrate 200mg/kg, LA: Rats orally received lead acetate 50 mg/kg, SB+LA: Rats orally received both sodium butyrate200mg\kg and Lead acetate 50mg\kg.

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Lead acetate exposure considerably enhances AST and ALT in the body (Mohammed, 2010), which can be attributed to an increase in oxidative stress caused by this exposure. The AST-ALT enzymes are special enzymes that can be used to identify liver disease. Those enzymes, commonly found in the cytoplasm of liver cells, leak into the extracellular fluid when membrane permeability is disrupted. However, in case of tissue damage, the cells will rupture, and the enzymes will break down from hepatocytes into the circulatory system, causing their levels in the blood to rise compared to the normal state (Chang et al., 2013). According to previous research, lead exposure is linked to liver damage and increased transaminases and ALP (Shirazinia et al., 2021). In a mouse model of non-alcoholic steatohepatitis (NASH), researchers found that 6 weeks of SB supplementation (0.6 g/kg body weight per day in a liquid diet) prevented the development of NASH, as evidenced by fewer fat-laden hepatocytes, lower expression of pro-inflammatory genes in the liver, and improved insulin signaling compared to untreated control mice (Jin et al., 2016).

Under the effect of lead, oxidative stress manifests itself in two ways of the production of reactive oxygen species (ROS) and the depletion of antioxidant reserves (Flora et al., 2012). Oxidative stress occurs when the body's antioxidant enzymes are depleted and the formation of free radicals. As polyunsaturated fatty acids (PUFA) undergo an increased process of lipid peroxidation, ROS attack all cellular structures, resulting in the loss of cellular and mitochondrial membrane integrity (Jackie et al., 2011). Malondialdehyde is a byproduct of PUFA peroxidation that causes cell membrane damage (Yuniarti et al., 2021). It is also one of the indicators utilized in clinical settings to measure oxidative stress levels. Increased MDA levels in the liver indicate increased lipid peroxidation, which results in tissue damage and the failure of antioxidant mechanisms to prevent the generation of excessive free radicals (Koerniasari et al., 2015).

Furthermore, the current study revealed that pretreatment with SB (200 mg/kg) reduces MDA while increasing superoxide dismutase levels (a key antioxidant enzyme). Furthermore, it was shown that antioxidants reduce HMGB1 expression and prevent pancreatic injury in rats, demonstrating that the release of HMGB1 from cultured hepatocytes is a dynamic process regulated by reactive oxygen species (Zhang et al., 2010). Overproduction of oxygen ROS during oxidative stress can result in lipid peroxidation, protein degradation, and DNA damage (Carocho and Ferreira, 2013). Since mitochondria is the primary source of ROS, it can be targeted by sodium butyrate's antioxidant activity (Zhang et al., 2020). Therefore, under oxidative stress conditions, mitochondria serve as both a substantial generator of ROS and a significant target for oxidative damage (Cao et al., 2020). Impaired mitochondria caused by oxidative stress can release 10 times the amount of ROS as normal mitochondria. The body will use lysosome breakdown to selectively remove damaged mitochondria, a process known as mitophagy (Wang and Klionsky, 2011).

Hepatic cell cancer cell proliferation can be effectively inhibited by SB (Seong and Lee, 2012). The cell cycle's arrest probably explains this effect in the G1 phase, and the decrease in the number of S-phase cells (Xu et al., 2020). The cyclin-dependent kinase inhibitors Cyclin-dependent kinase inhibitors p21 and p27 are crucial for stopping the cell cycle in the G1 phase (Izutani et al., 2012).

In the current report, SB treatment gene expression data seemed to indicate that high expression of anti-inflammatory T-cell factors PPAR-g and IL-10 improved patient inflammation. Increasing PPAR-γ expression and nuclear factor-κB downregulation results suggested that oral supplementation of butyrate could be beneficial as an adjuvant in treating obesity, metabolic syndrome, and insulin resistance (Wen et al., 2021). The production of pro-inflammatory cytokines like TNF-, IL-1, and IL-6 is dose-dependently inhibited by PPAR-agonists (Jiang et al., 1998).

It was indicated that the expression of PPAR-g was closely correlated with the transcriptional activity of IL-10 (Yang et al., 2010). The PPAR-g activation results in Granulocyte colony-stimulating factor production and subsequently Myeloid-derived suppressor cell mobilization (Hegde et al., 2015). There is evidence that PPAR-g plays a key role in regulating Treg cell accumulation and function (Cipolletta et al., 2015). Sodium butyrate treatment can activate PPAR-g signaling, and consequently inducing the Treg cells and MDSCs, which are anti-inflammatory cells and regulate the immune response to decrease lead acetate poising.

CONCLUSION

Sodium butyrate may be considered an antioxidant and has positive effects on liver function. It is therefore suggested to consider the analysis of different doses of sodium butyrate supplements on brain or other soft tissues.

DECLARATIONS

Acknowledgments

This study was funded by a physiology laboratory, and the animals were acclimatized in the animal home of the University of Baghdad's College of Veterinary Medicine, Baghdad, Iraq.

Authors' contribution

Amira Kamil Mohammed, designed all the experiments and Rusul Mowaffaq Ahmed performed all experiments under the supervision of Amira Kamil Mohammed. Rusul Mowaffaq Ahmed collected the data and wrote the draft of the manuscript. Amira Kamil Mohammed contributed to analyzing the data. Amira Kamil Mohammed supervised the work

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from designing to finalizing the manuscript for journal submission. All authors checked and approved the final version of the manuscript for publishing in the present journal

Competing interests

There is no conflict of interest.

Ethical consideration

All of the authors have reviewed the manuscripts for ethical concerns, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, duplication of publication and/or submission, and redundancy.

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Antimicrobial Effects of Selenium and Chitosan Nanoparticles on Raw Milk and Kareish Cheese

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ABSTRACT

The contamination of milk and its dairy products with different microorganisms could cause public health hazards. Antibacterial nanoparticles (NPs) are a novel way to ensure that milk and milk products are safe. The present study investigated the effect of chitosan NPs (CS-NPs) and selenium NPs (Se-NPs) on some microorganisms, which consequently affect raw milk and Kareish cheese. Small-sized nanomaterials of Se-NPs and CS-NPs at the size of approximately 20 nm were used in this study. The samples were 700 ml raw milk and 700g Kareish cheese manufactured from 3000 mg milk. The concentrations of used nanoparticles were 0.5%, 1%, and 1.5% for Se-NPs and 2.5%, 5%, and 10% for CS-NPs. They were used to improve the microbial properties of milk and Kareish cheese samples during storage at the refrigerated temperature of 4°C. The aerobic plate count, Enterobacteriaceae count, Staphylococcus count, and mold count were significantly reduced in milk and Kareish cheese samples treated with CS-NPs and Se-NPs. The study has confirmed that CS-NPs and Se-NPs indicated high antimicrobial activity against the studied microorganisms at all concentrations although CS-NPs were more effective than Se-NPs. It can be concluded that these NPs can be used as preservatives in milk and milk products, such as Kareish cheese. In addition, increasing the concentrations of these NPs by 10% for CS-NPS and 1.5% for Se-NPS boosted their effects.

Keywords: Chitosan, *Enterobacteriaceae*, Kareish cheese, Nanoparticle, Selenium, *Staphylococcus aureus*

INTRODUCTION

In many parts of the world, milk and dairy products are perfect media for microorganism growth due to their high nutritional content (Ledenbach and Marshall, 2009). It is impossible to eliminate microbe contamination of milk during the preparation of various dairy products; consequently, the microbiological content of milk is an important factor in its quality from a safety standpoint (Singh et al., 2011). Many zoonotic bacteria, such as *Escherichia coli* (*E. coli*), *Salmonella*, and *Staphylococcus aureus* (*S. aureus*), can be found in dairy products and can cause serious diseases, especially in immunocompromised consumers (Pal, 2007).

Nanotechnology has made its way into improving the quality of food as well as unique food supplements, additives, and nutrients (Huang et al., 2017). It is a new technology that could mark the beginning of the second technological generation. It focuses on the characterization, fabrication, and manipulation of structures or materials smaller than 100 nm (Ozimek et al., 2010). It aims to improve the tastes, textures, and bioavailability of minerals and supplements, as well as extending the shelf life of the products (Chaudhry and Castle, 2011). As a result, nanotechnological advantages have lately been used to tackle food and environmental challenges **(**Jaiswal et al., 2019) by enhancing the quality of micronutrients during processing, storage, and distribution (Chen et al., 2006). Nanomaterials are now used in the food industry for various purposes, such as food ingredients or additives, or as part of packaging materials (Rhim et al., 2013). Selenium nanoparticles (Se-NPs) can be used instead of antibiotics, such as ampicillin, to prevent and treat a variety of bacterial diseases and infections in people. Nano selenium is 60 times more effective than traditional treatments in treating infections caused by *S. aureus*, *E. coli*, and *P. aeruginosa*.

It can improve absorption into plants, animals, people, and microbes and act as an antioxidant with a lower risk of selenium toxicity. Furthermore, one of the most important uses of Se-NPs is chemoprevention via immune activation (Majeed et al., 2018). Chitin is a polysaccharide of animal origin that has a fibrous structure and is abundant in nature. Chitosan (CS) can be made by removing the acetyl groups from the chitin structure that is the major component of the exterior skeleton of insects and crustaceans, such as shrimp, crabs, and lobster (Kumar et al., 2005). Because of its nontoxicity, biodegradability, and antibacterial characteristics (Widnyana et al., 2021), CS is used in biomedical research, agriculture, genetic engineering, as well as the food industry, and water treatment (El-Dahma et al., 2017). Chitosan has a stronger effect on Gram-positive bacteria (*S. aureus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and

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Lactobacillus bulgaris) than Gram-negative bacteria (*E. coli*, *Salmonella typhy* (Coma et al., 2003). In contrast, Chung et al. (2004) found that Gram-negative bacteria are more sensitive to CS than Gram-positive bacteria as they have substantially more hydrophilicity. Chitosan's antifungal effect is thought to be fungistatic rather than fungicidal, with the potential to transmit regulatory changes in both the host and the fungus (Raafat and sahl, 2009).

This study aimed to investigate the way CS-NPS and Se-NPs effectively reduce pathogens in milk and Kareish cheese during cold storage.

MATERIAL AND METHODS

Collection of samples

Fresh raw milk (700ml) used in this study was purchased from dairy shops in El Monofiya Governorate, Egypt. All samples were kept in an ice box, transferred to the laboratory with minimum delay under completely hygienic conditions, and examined as rapidly as possible. The total sample was divided into two parts, one for raw milk examination and the other for manufacturing Kareish cheese for cheese examination. The experiment was repeated three times on different batches of milk dairy shops.

Preparation of milk sample

The milk samples (700 ml of raw milk) were divided into seven groups, 100 ml per group. The first three groups were treated with Se-NPs at concentrations of 0.5%, 1%, and 1.5%. The second three groups were treated with 2.5%, 5%, and 10% CS-NPs. The seventh group served as control. All samples were kept at 4°C. The analysis of the samples was performed on at days 1, 3, 6, 9, 12, and 15 of storage.

Kareish cheese manufacturing

Cheese manufacture essentially involves the coagulation of casein. Raw milk was heated at 74°C for 15 seconds and then cooled rapidly to 40°C AQt this point, 1.5% yogurt starter culture was added for coagulation. When coagulation had been completed, the curd was transferred into gauze to get rid of whey in 24 hours. In the next step, cut and stored in its pasteurized salted whey (7% salt) for 24 hours. Cheese samples were stored at 4°C (Phelan et al., 1993). In Kareish cheese, the fat level in dry matter and the moisture content should not exceed 10% and 75%, respectively (Egyptian Standard 2000/4-1008). To manufacture 700g of Kareish cheese, 3000 ml of raw milk was used. After that, the cheese was divided into seven groups followed by the addition of Se-NPs (0.5%, 1%, and 1.5%) and CS-NPs (2.5%, 0.5%, and 1%).

Nanomaterials

The Se-NPs and CS-NPs were prepared at the Naqaa Foundation for Scientific Research, Technology, and Development in Giza, Egypt. The Se-NPs were prepared according to the modified method of Qian Li et al. (2010). The Se solution was obtained by adding 100 mM of Sodium selenite to 50 mM ascorbic acid. Varied sodium selenite to ascorbic acid ratios (1:1, 1:2, 1:3, 1:4, 1:5, 1:6) had been reacted from the stock solution. The ascorbic acid was added drop by drop to the sodium selenite under magnetic stirring at various rpm (200, 600, 1000 rpm) at room temperature for 30 minutes. Combos had been allowed to react with each other's in the targeted shape until the shade alternate was observed from colorless to mild orange. Soon after the shad alternate was once determined, the combination used to be diluted to 25 ml with double distilled water.

Chitosan NPs were prepared according to Calvo et al. (1998). Chitosan deacetylation was 75%, with a molecular weight of 200 KDa. The CS solution was made using the ionotropic gelation process, which involved dissolving 100 mg of CS in a 1 percent v/v acetic acid solution and stirring it at room temperature until it turned transparent. A 0.1 molar sodium hydroxide solution was added to the mixture with a pH of 6.5. In a Pyrex glass flask, 10 ml of 0.80 mg/ml tripolyphosphate aqueous solution was added dropwise at room temperature under a magnetic stirrer at 750 rpm. The solution was then sonicated at room temperature for 10 minutes at 80 percent amplitude using the SB-5200 DTD Ultrasonic Cleaner, China (Vaezifar, 2013). The CS-NP solution was filtered by nylon syringe 0.22µm mesh and then freeze-dried for subsequent analysis.

Microbiological assay

Serial dilution was prepared according to ISO 1999; the surface plate method determined aerobic plate count at 35°C (Petran et al., 2015). After that, Baird Parker Agar was used to isolate and differentiate coagulase-positive *staphylococci* in food (FDA, 2001). Colonies appeared in gray-black, and a clear halo was developed around colonies from coagulase-positive *S. aureus*. Enterobacteriaceae counts, *E coli*, and *Salmonella spp.*, were determined according to ISO 21528: 2017. Neutral red colonies resulted in pink colonies due to glucose fermentation resulting from produced acid and decreased PH. Finally, Enumeration and isolation of fungi were done according to International Commission on

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Microbiological Specifications for Foods (ICMSF, 1996) using Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml), which was then incubated at 28-30°C for 2-21 days.

Statistical analysis

Microbiological data were converted into logarithms of the colony number of forming units (CFU/gm). The analysis of variance (ANOVA) was performed in SPSS software (Version 22, SPSS Inc. Chicago, IL, and USA). Means and standard deviations were calculated. By applying Duncan's Multiple Range test, multiple mean comparisons were made to measure the specific differences between pairs of means. Values were statistically significant at the $p \leq 0.05$ level

RESULTS AND DISCUSSION

Selenium has an antibacterial effect at extremely high concentrations (1.5 to 3 mg/kg body weight), which are fatal to living organisms. Therefore, its use is limited to medicinal purposes (Khiralla and El-Deeb 2015). According to some researchers, Se-NPs are better than elemental selenium because they have antibacterial activity at low doses of 20 µg/mL (Huang et al., 2017).

Because of its nontoxicity, biodegradability, and antibacterial characteristics, CS is used for various purposes (Widnyana et al., 2021), including food processing (Cheba, 2011). *In vitro* tests have shown that Gram-negative bacteria are more sensitive to CS than Gram-positive bacteria, with higher morphological alterations after treatment (Chen et al., 2002; Simunek et al., 2006; and Eaton et al., 2008). The amount of adsorbed CS is determined by the charge density on the cell surface. Adsorbed CS at higher levels would cause more cell membrane structure and permeability alterations. This indicates that the host-microbe influences the antibacterial method of action (Másson et al., 2008). In the current study, the microbiological changes as aerobic plate count (APC), *Staphylococcus* spp. count, Enterobacteriaceae count, and mold count of milk and cheese samples were estimated throughout the cooling storage at 4°C for 15 days

Aerobic bacterial count

Aerobic bacterial count in milk samples

The initial total bacterial load was reduced over time when CS-NPs and Se-NPs were added to milk samples. During chilling storage of milk samples treated with CS-NPs, APC decreased from 5.71 to 4.2 ~1 $\log_{10}$CFU/ml at a concentration of 2.5%. The microbial effect of CS-NPs against total bacterial count increased by increasing the concentration of CS-NP**s**, so when the concentration reached 10%, the count of total bacterial count significantly decreased from 5.71 to 3.86 (~2 $\log_{10}$) CFU/ml (Table 1, $p \leq 0.05$). In milk samples treated with 0.5% Se-NPs, APC decreased from 5.71 to 4.2 ~1 $\log_{10}$ CFU/ml, but when the concentration reached 1.5%, APC decreased from 5.71 to 3.8 ~2 $\log_{10}$ CFU/ml ($p \leq 0.05$, Table 1). The Egyptian standards for raw milk (ES:154-1/2005) mentioned that the acceptable count of total bacterial count should be less than 200 count /ml (EOS, 2005)**.**

Aerobic bacterial count in Kareish cheese samples

In Kareish cheese samples treated with 10% CS-NPs, the APC count decreased from 4.56 to 2.63 ~2 $\log_{10}$ CFU/ml (Table 2) and the APC count significantly decreased from 4.56 to 3.4 ~1 $\log_{10}$ CFU/ml in cheese samples treated with 1.5% Se-NPS (Table 2, $p \leq 0.05$). According to the obtained results, 10% CS-NPs was the most effective antimicrobial agent (against total bacterial count followed by 1.5% Se-NPs. As Hariharan et al. (2012) stated, the antibacterial activity was related to the concentration of nanoparticles. Gram-positive and Gram-negative bacteria are both inhibited by the antibacterial mechanism of Se-NPs, which is unknown yet. Currently, it is thought that Se-NPs break the bacterial cell wall by interacting with the peptidoglycan layer and damaging the double-stranded DNA structure (Sonkusre et al., 2014). Chitosan NPs have antimicrobial activity due to electrostatic interaction between their positive charge and the negative charge of bacterial membranes leading to cell membrane lysis of bacterial cells (Rabea et al., 2003; Tripathi et al., 2008). Chitosan NPs can also interact with essential microbial nutrients, causing microbial growth disruption and eventually death (Jia et al., 2001; Rabea et al., 2003).

***Staphylococci* bacterial count**

As shown in figures 1 and 2, the antibacterial effect of nanoparticles against staphylococci in milk and Kareish cheese, respectively, was confirmed.

Staphylococci count in milk samples

Staphylococci count decreased in milk samples from 4.66 to 3.36 ~1 $\log_{10}$ CFU/ml at the concentration of 2.5% CS-NPs. Moreover, the microbial effect of CS-NPs against *staphylococci* increased by increasing the concentration of CS-NP**s,** so the count of *staphylococci* decreased from 4.66 to 2.66 (~2 $\log_{10}$) CFU/ml when the concentration reached 10% (Figure 1). In milk samples treated with 0.5% Se-NPs, *staphylococci* decreased from 4.66 to 3.3 ~1 $\log_{10}$ CFU/ml, and when the concentration reached 1.5%, *staphylococci* decreased from 4.66 to 2.7 ~2 $\log_{10}$ CFU/ml (Figure 1).

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Table 1. Effects of different concentrations of chitosan and selenium nanoparticles on the aerobic plate count of the examined milk samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	5.71 ± 0.05^{a}	5.84 ± 0.089^{b}	6.48 ± 0.094 ^{d}	6.87 ± 0.061^{d}	7.51 ± 0.047^{d}	7.88 ± 0.04^{d}
2.5% chitosan	5.71 ± 0.05^{a}	5.57 ± 0.02^{ab}	5.25 ± 0.031^{ab}	4.84 ± 0.01^{e}	4.61 ± 0.02^{e}	4.2 ± 0.03^{f}
5% chitosan	5.71 ± 0.05^{a}	5.43 ± 0.03^{ab}	5.19 ± 0.02^{ab}	4.72 ± 0.01^{e}	4.27 ± 0.01^{f}	3.86 ± 0.04^{fg}
10% chitosan	5.71 ± 0.05^{a}	5.11 ± 0.035^{ab}	4.86 ± 0.03^{c}	4.5 ± 0.01^{f}	3.74 ± 0.04^{fg}	3.17 ± 0.05^{g}
0.5% selenium	5.71 ± 0.05^{a}	5.67 ± 0.01^{ab}	5.46 ± 0.06^{ab}	4.91 ± 0.03^{e}	4.80 ± 0.02^{e}	4.62 ± 0.02^{f}
1% selenium	5.71 ± 0.05^{a}	5.58 ± 0.01^{ab}	5.43 ± 0.01^{ab}	4.79 ± 0.02^{e}	4.53 ± 0.01^{f}	4.39 ± 0.01^{fg}
1.5% selenium	5.71 ± 0.05^{a}	5.38 ± 0.03^{ab}	5.17 ± 0.02^{ab}	4.67 ± 0.01^{e}	4.38 ± 0.01^{f}	3.8 ± 0.07^{fg}

The values represented as mean ± standard deviation of three experiments. $^{a, b,c, d,e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

Table 2. Effect of different concentrations of chitosan and selenium nanoparticles on the aerobic plate count of the examined cheese samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	4.56 ± 0.3^{a}	4.76 ± 0.3^{b}	4.98 ± 0.3^{e}	5.39 ± 0.13^{e}	5.56 ± 0.13^{g}	6.3 ± 0.4^{g}
2.5% chitosan	4.56 ± 0.3 ^{a}	4.37 ± 0.2^{ab}	4.22 ± 0.15^{c}	3.85 ± .02^{d}	3.45 ± 0.1^{d}	3.33 ± 0.11^{d}
5% chitosan	4.56 ± 0.3^{a}	4.31 ± 0.11^{ab}	4.19 ± 0.11^{cd}	3.63 ± 0.1^{d}	3.4 ± 0.17^{d}	3.32 ± 0.16^{d}
10% chitosan	4.56 ± 0.3 ^{a}	4.24 ± 0.2^{ab}	4.15 ± 0.21^{d}	3.42 ± 0.15^{d}	3.25 ± 0.14^{f}	2.63 ± 0.2^{h}
0.5% selenium	4.56 ± 0.3 ^{a}	4.45 ± 0.15^{ab}	4.37 ± 0.2^{c}	4.25 ± 0.2^{c}	4.13 ± 0.1^{c}	3.82 ± 0.13^{d}
1% selenium	4.56 ± 0.3 ^{a}	4.4 ± 0.1^{ab}	4.28 ± 0.1^{cd}	4.13 ± 0.3^{c}	3.85 ± 0.3^{d}	3.6 ± 0.11^{d}
1.5% selenium	4.56 ± 0.3 ^{a}	4.32 ± 0.2^{ab}	4.22 ± 0.1^{cd}	3.91 ± 0.2^{cd}	3.61 ± 0.1^{d}	3.41 ± 0.20^{d}

The values represented as mean ± standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

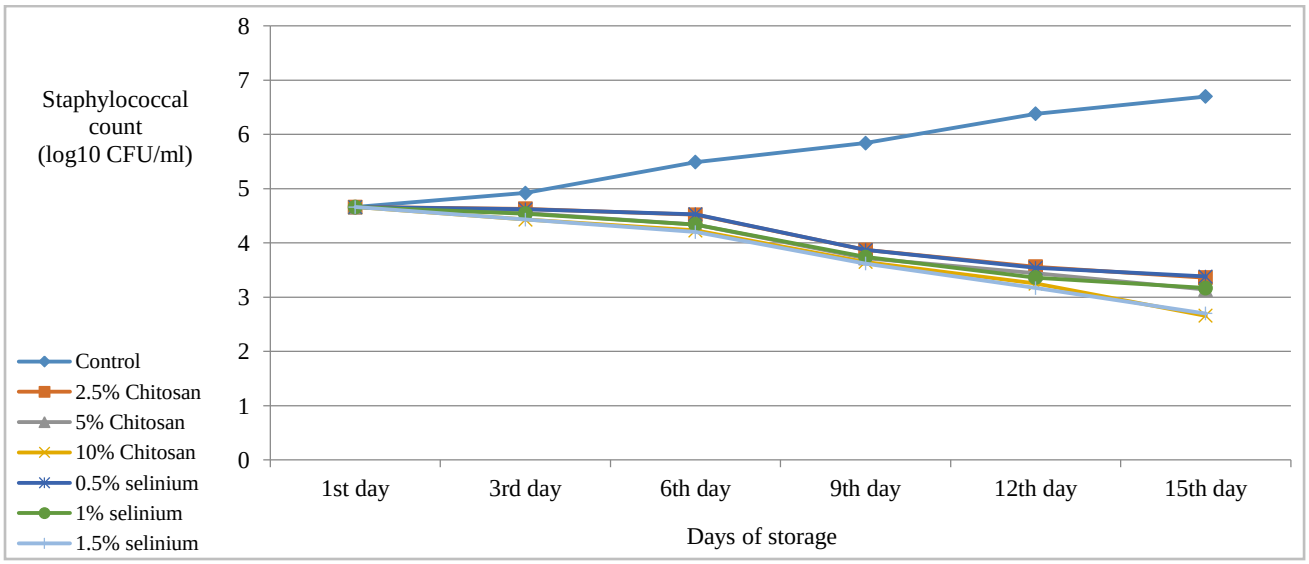

Figure 1. Effect of different concentrations of chitosan and selenium nanoparticles on *Staphylococci* count of the examined milk samples during storage at 4ºC

Staphylococci count in Kareish cheese samples

Staphylococci count decreased in cheese samples from 4.43 to 3.24 ~1 $\log_{10}$CFU/ml using 2.5% CS-NPs. The microbial effect of CS-NPs against *staphylococci* increased by increasing the concentration of CS-NP**s,** so when the concentration reached 10%, the count of *staphylococci* decreased from 4.43 to 2.58 ~2 $\log_{10}$ CFU/ml (Figure 2). In cheese samples treated with Se-Nps at the concentration of 0.5%, *staphylococci* decreased from 4.43 to 3.63~1 $\log_{10}$ CFU/ml, and when the concentration reached 1.5%, *staphylococci* decreased from 4.43 to 3.2 ~1 $\log_{10}$ CFU/ml as seen in Figure 2. Similar to the findings of Qi et al. (2004), Ro-drigus-Nunez et al. (2012), Salmabi and Seema (2013), Van Toan et al. (2013), Younes et al. (2014), and Widnyana et al. (2021), the *S. aureus* was inhibited by CS. Moreover, the antimicrobial effect of Se-NPs recorded by Khiralla and El-Deeb (2015) indicated that the inhibition zone increased with an increase in the concentration of Se-NPs. The Se-NPs were reported to be a potent antimicrobial agent against *S. aureus* (Chudobova et al., 2014). According to Phong et al. (2011), the proportion of live *S. aureus* decreased in the presence of Se-NPs at 7.8, 15.5, and 31 g/mL after 3, 4, and 5 hours. The Egyptian standards for Kareish cheese (No.1008/2000) mentioned that *S. aureus* (coagulate-positive) was absent in 1 g (EOS, 2000).

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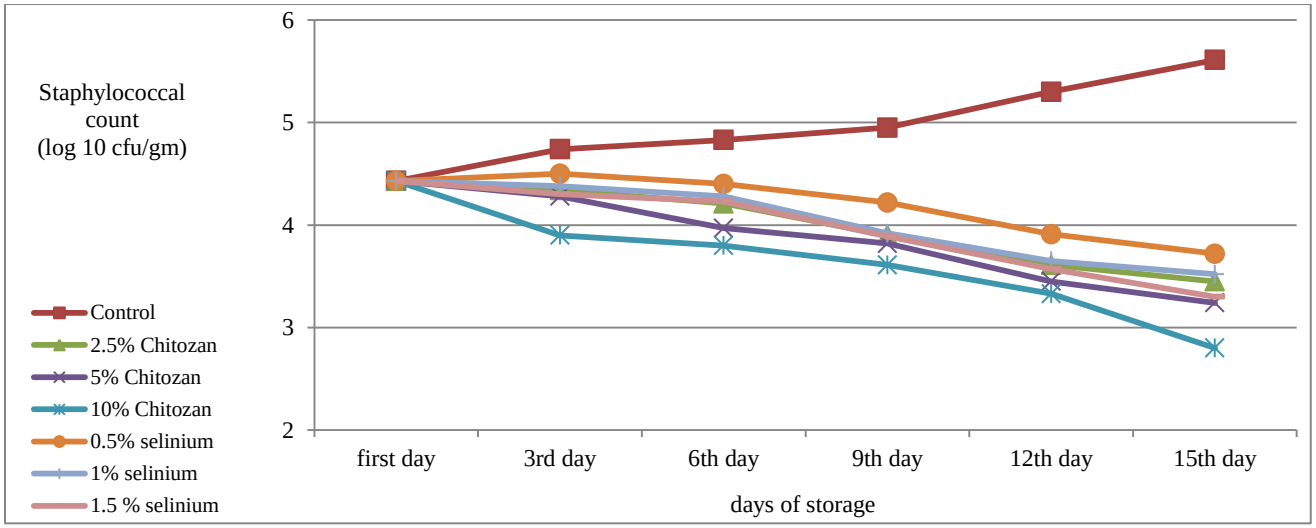

Figure 2. Effect of different concentrations of chitosan and selenium nanoparticles on *Staphylococci* count of the examined cheese samples during storage at 4ºC

Enterobacteriaceae count

Tables 3 and 4 showed that results obtained from treated samples significantly differed from the control result ($p \leq 0.05$). The antibacterial and antibacterial assays of CS-NP**s** against Gram-negative and Gram-positive bacteria were applied at different concentrations. The inhibition increased by increasing the concentration of CS-NP**s**. Gram-negative bacteria were more sensitive to CS-NP**s** than gram-positive bacteria (Coma et al., 2003).

Enterobacteriaceae count in milk samples

Enterobacteriaceae count has been decreased in milk samples from 4.57 to 3.27 ~1 $\log_{10}$CFU/ml at a concentration of 2.5%, and the microbial effect of CS-NPs against Enterobacteriaceae increased by increasing the concentration of CS-NP**s**. Enterobacteriaceae were not detected at the highest concentration of chitosan, 10% (Table 3). In milk samples treated with 0.5% Se-Nps, Enterobacteriaceae decreased from 4.57 to 3.39 ~1 $\log_{10}$ CFU/ml, but when the concentration reached 1.5%, *staphylococci* decreased from 4.57 to 2.65 ~1 $\log_{10}$ CFU/ml (Table 3). The Egyptian standards for raw milk (ES:154-1/2005) declared that the total coliforms are less than 10 count/ml *E. coli* and *Salmonella* and other pathogens absent in 1 ml (EOS, 2005).

Enterobacteriaceae count in Kareish cheese samples

Enterobacteriaceae count significantly decreased in cheese samples from 3.44 to 2.63 ~1 $\log_{10}$CFU/ml in different concentrations of CS-NP**s** and Se-Nps ($p \leq 0.05$, Table 4). The results agreed with Balicka-Ramisz et al. (2005), Liu et al. (2006), and Chung and Chen (2008), who reported that CS had antibacterial activity against *E. coli*. Balicka-Ramisz et al. (2005) and Benhabiles et al. (2012) recorded the antibacterial activities of CS against *Salmonella* sp. Results agreed with Hassanien and Shaker (2020), who used CS-NPs at a 30 µg/mL concentration. Chitosan NPs exerted a high bactericidal effect on isolates, such as *E. coli O157:H7* recovered from Kareish cheese samples, which significantly increased with an increase in concentration. Khiralla and El-Deeb (2015) evaluated the effect of Se-NPs against foodborne pathogens, such as *E. coli* and *S. aureus*. They found that the inhibition zone increases with increasing Se-Nps concentration. According to Shrestha et al. (2010) and Khurana et al. (2019), CS-NPs and Se-NPs have antibacterial effects against *Enterococcus faecalis*. Selenium NPs were highly effective against *E. faecalis* biofilm at the concentration of 1mg/ml (Sanjay et al., 2021). The Egyptian standards for Kareish cheese (No.1008/2000) mentioned that the total coliforms should be less than 10 CFU/g, *E. coli* should be absent in 1g Kareish cheese, *Salmonella* and other pathogens absent in 25 g (EOS, 2000).

Molds count

The antibacterial action of nanoparticles against molds was demonstrated by the fact that the counts of treated and control samples were significantly different ($p \leq 0.05$)**.** Molds were not detected at high concentrations of CS-NPs (10%) and Se-NPs (1.5%) in milk samples (Table 5). However, the mold count significantly decreased by ~1 $\log_{10}$ CFU/ml in cheese samples at high concentrations CS-NPs (10%) and Se-NPs (1.5%), respectively ($p \leq 0.05$, Table 6). Antifungal activity in the current study agreed with that of Yien et al. (2012), indicating that the CS-NP**s** were observed to be natural antifungal agents when used in concentrations of 1-3 mg/ml against *Candida albicans*, *Aspergillus niger*, and *Fusarium solani* pathogenic strain isolated from clinical specimens. Moreover, a study by Shakibaie et al. (2015) indicated the anti-biofilm activity of biologically generated (Se-NPs) in concentrations ranging from 10 to 200 mg/mL against the biofilm

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produced by clinically isolated fungus strains, such as *Aspergillus fumigatus* and *Candida albicans*. The obtained results of the current study agreed with those of Rasha et al. (2019), indicating that the use of CS-NPS in concentrations of 0.25% and 0.5% before or after manufacturing Kareish cheese could prolong safe preservation as the nano-chitosan have the antimicrobial potential against several bacteria and fungi, such as *Aspergillus flavus*. Furthermore, Elsharawy et al. (2019) revealed that the mold counts in Kareish cheese treated with 1% CS were lower than untreated cheese samples during the storage period. Since direct contact with CS causes hyphae to weaken and swell, the fungistatic characteristics of CS are linked to its ability to induce morphological changes in the cell wall (Rabea et al., 2003). The Egyptian standards for Kareish cheese (No.1008/2000**)** mentioned that yeasts and molds should be less than 10 CFU/g (EOS, 2000).

Table 3. Effect of different concentrations of chitosan and selenium nanoparticles on Enterobacteriaceae count of the examined milk samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	$4.57 \pm 0.0\ 2^{a}$	4.76 ± 0.09^{a}	4.97 ± 0.04^{a}	5.53 ± 0.06^{a}	5.89 ± 0.05^{e}	6.2 ± 0.04^{g}
2.5% chitosan	$4.57 \pm 0.\ 02^{a}$	4.17 ± 0.02^{a}	3.92 ± 0.031^{b}	3.79 ± 0.01^{c}	3.65 ± 0.02^{c}	3.27 ± 0.03^{bc}
5% chitosan	$4.57 \pm 0.\ 02^{a}$	4.34 ± 0.03^{a}	3.73 ± 0.02^{c}	3.11 ± 0.01^{bc}	3.14 ± 0.01^{dc}	2.75 ± 0.04^{f}
10% chitosan	$4.57 \pm 0.\ 02^{a}$	4.19 ± 0.04^{b}	3.36 ± 0.03^{bc}	2.82 ± 0.01^{d}	2.74 ± 0.04^{f}	*ND
0.5% selenium	$4.57 \pm 0.0\ 2^{a}$	4.49 ± 0.01^{a}	4.17 ± 0.06^{b}	3.9 ± 0.03^{c}	3.87 ± 0.02^{c}	3.39 ± 0.02^{c}
1 % selenium	$4.57 \pm 0.\ 02^{a}$	4.36 ± 0.01^{a}	3.76 ± 0.01^{c}	3.44 ± 0.02^{c}	3.57 ± 0.01^{dc}	2.95 ± 0.01^{f}
1.5 % selenium	4.57 ± 0.02^{a}	4.23 ± 0.03^{b}	3.57 ± 0.02^{c}	3.35 ± 0.01^{d}	2.9 ± 0.01^{f}	2.65 ± 0.07^{f}

The values represented as mean ± standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$). *ND: Not detected

Table 4. Effect of different concentrations of chitosan and selenium nanoparticles on Enterobacteriaceae count of the examined cheese samples during storage at 4ºC.

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	3.44 ± 0.1^{a}	3.56 ± 0.3^{a}	3.98 ± 0.1^{e}	4.26 ± 0.13^{e}	4.56 ± 0.13^{f}	4.73 ± 0.2^{f}
2.5% chitosan	$3.44 \pm 0.1\ ^{a}$	3.33 ± 0.2^{ab}	3.27 ± 0.1^{b}	$3.12 \pm .02^{c}$	2.91 ± 0.1^{cd}	2.63 ± 0.11^{d}
5% chitosan	$3.44 \pm 0.1\ ^{a}$	3.28 ± 0.1^{ab}	3.21 ± 0.1^{b}	2.85 ± 0.2^{cd}	2.66 ± 0.2^{d}	2.42 ± 0.14^{d}
10% chitosan	3.44 ± 0.1^{a}	3.2 ± 0.1^{ab}	3.15 ± 0.1^{c}	2.69 ± 0.15^{d}	2.52 ± 0.14^{d}	2.32 ± 0.2^{g}
0.5% selenium	3.44 ± 0.1^{a}	3.4 ± 0.2^{ab}	3.33 ± 0.2^{b}	3.22 ± 0.2^{c}	3.12 ± 0.1^{c}	2.83 ± 0.15^{cd}
1% selenium	3.44 ± 0.1^{a}	3.35 ± 0.1^{ab}	3.29 ± 0.1^{b}	3.13 ± 0.3^{c}	2.84 ± 0.1^{cd}	2.64 ± 0.21^{cd}
1.5% selenium	3.44 ± 0.1^{a}	3.3 ± 0.2^{ab}	3.25 ± 0.1^{b}	2.98 ± 0.2^{cd}	2.72 ± 0.1^{cd}	2.58 ± 0.23^{d}

The values represented as mean ± standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

Table 5. Effect of different concentrations of chitosan and selenium nanoparticles on molds count of the examined milk samples during storage at 4ºC.

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	3.8 ± 0.04^{a}	3.96 ± 0.01^{a}	4.73 ± 0.09^{e}	4.95 ± 0.061^{e}	5.67 ± 0.04^{g}	5.92 ± 0.04^{g}
2.5% chitosan	3.8 ± 0.04^{a}	3.51 ± 0.02^{a}	3.35 ± 0.031^{ab}	3.22 ± 0.01^{c}	2.81 ± 0.02^{d}	2.3 ± 0.03^{d}
5% chitosan	3.8 ± 0.04^{a}	3.36 ± 0.03^{ab}	3.21 ± 0.02^{ab}	2.7 ± 0.01^{cd}	2.63 ± 0.01^{d}	*ND
10% chitosan	3.8 ± 0.04^{a}	3.11 ± 0.03^{b}	2.9 ± 0.03^{c}	2.59 ± 0.01^{d}	2.35 ± 0.04^{f}	*ND
0.5% selenium	3.8 ± 0.04^{a}	3.75 ± 0.01^{a}	3.67 ± 0.06^{ab}	3.41 ± 0.03^{c}	3.11 ± 0.02^{cd}	2.7 ± 0.02^{d}
1% selenium	3.8 ± 0.04^{a}	3.68 ± 0.01^{a}b	3.56 ± 0.01^{ab}	3.28 ± 0.02^{cd}	2.94 ± 0.01^{d}	*ND
1.5% selenium	3.8 ± 0.04^{a}	3.59 ± 0.03^{ab}	3.34 ± 0.02^{ab}	2.91 ± 0.01^{cd}	2.55 ± 0.01^{d}	*ND

The values represented as mean ± standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$). *ND: Not detected.

Table 6. Effect of different concentrations of chitosan and selenium nanoparticles on molds count of the examined cheese samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	3.54 ± 0.3^{a}	3.73 ± 0.2^{a}	3.92 ± 0.21^{e}	4.34 ± 0.13^{e}	4.57 ± 0.2^{f}	5.35 ± 0.3^{f}
2.5% chitosan	3.54 ± 0.3^{a}	3.43 ± 0.14^{ab}	3.33 ± 0.15^{b}	3.22 ± 0.2^{c}	2.83 ± 0.12^{cd}	2.63 ± 0.12^{d}
5% chitosan	3.54 ± 0.3^{a}	3.36 ± 0.12^{ab}	3.26 ± 0.11^{b}	3.13 ± 0.11^{cd}	2.72 ± 0.15^{d}	2.53 ± 0.1^{d}
10% chitosan	3.54 ± 0.3^{a}	3.24 ± 0.2^{ab}	3.16 ± 0.14^{c}	2.77 ± 0.12^{d}	2.53 ± 0.13^{d}	2.42 ± 0.14^{g}
0.5% selenium	3.54 ± 0.3^{a}	3.48 ± 0.13^{ab}	3.4 ± 0.21^{b}	3.32 ± 0.14c	3.13 ± 0.1^{c}	2.76 ± 0.11^{cd}
1% selenium	3.54 ± 0.3^{a}	3.39 ± 0.11^{ab}	3.31 ± 0.11^{b}	3.15 ± 0.20^{c}	2.92 ± 0.14^{cd}	2.68 ± 0.2^{cd}
1.5% selenium	3.54 ± 0.3^{a}	3.3 ± 0.21^{ab}	3.25 ± 0.2^{b}	2.91 ± 0.21^{d}	2.65 ± 0.1^{cd}	2.52 ± 0.13^{d}

The values represented as mean ± standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

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CONCLUSION

Biocompatible Se-NPs and CS-NPs had high antimicrobial activity against pathogenic and spoilage Gram-positive and Gram-negative bacteria, as well as molds that affect raw milk and Kareish cheese. According to this study, nanoparticles can be employed as a preservative in milk and Kareish cheese to extend their shelf life. Further studies should be conducted on the effectiveness of nanotechnology and nanoparticles on dairy products, their prevention of microbial contamination, and the limitation of mold excretions like aflatoxins.

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Authors' contribution

Hend Ahmed Elbarbary and Hamdy Abd El Samea Mohamed created the study plan and revised the research article. Shimaa Nabil Mohamed and Nahla Abo EL-Roos examined the data and conducted laboratory experiments. Shimaa Nabil did the statistical analysis and wrote the paper. Nahla Abo EL-Roos, who also revised the research manuscript, provided the experimental instruments. All authors read and approved the final version of the manuscript for publishing in the present journal

Competing interests

There are no conflicts of interest declared by the authors.

Ethical consideration

The author checked the manuscript for ethical issues, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publishing and/or submission, and redundancy.

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Basic Principles and Applications of Live Cell Microscopy Techniques: A Review

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ABSTRACT

Live cell imaging has provided great benefits in studying multiple processes and molecular interactions within and/or between cells. This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications. A wide range of microscopic techniques, from conventional transmitted light to an array of fluorescence microscopy techniques, including advanced super-resolution techniques, can be applied for live-cell imaging. Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. On the other hand, fluorescence microscopy uses reflected light to capture images of cells or molecules that have been fluorescently dyed. Techniques for transmitted light microscopy are simple to use but have poor resolution. Although the resolution of fluorescent microscopy techniques is only approximately 200-300 nm, this is nevertheless an improvement over conventional transmitted methods. Conventional light microscopy's resolution was improved by the introduction of the super-resolution microscopy technology family. These methods "break" the diffraction limit, enabling fluorescence imaging with resolutions up to ten times higher than those possible with traditional methods. Each live cell imaging method has advantages and drawbacks. The primary deciding criteria for choosing the type of microscope are the study's objectives, previous experience, the researcher's interests, and financial viability. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount in life science studies.

Keywords: Application, Fluorescence, Imaging, Laser-Scanning, Live cell, Microscopy

INTRODUCTION

Nowadays, live cell imaging is a very common and essential tool that is involved in illuminating a great deal about cellular dynamics and function (Frigault et al., 2009). It is crucial for conducting life science research in the fields of biology and pharmacology (Jensen, 2013). During live cell imaging studies on the lives of cells, it is very important to avoid altering the physiological and biological processes under investigation (Sanderson et al., 2014). Direct observation of the processes within a cell provides a crucial additional dimension to researchers' understanding of cell physiology and biology (Murphy and Davidson, 2012).

Cells are not usually exposed to light during their growth. It is, therefore, crucial to minimize light exposure of cells during microscopy applications by employing microscope systems optimized to collect as much light as possible using superior-quality optical components and detectors (Frigault et al., 2009). Bright-field microscopy is a traditional technique used to examine cells and tissues that usually requires fixation and subsequent staining before visualization under the microscope. It is impossible to obtain information from a living cell using this method since the fixation kills the cell; hence only a static, snapshot view of cells can be obtained (Mokobi, 2022). Currently, there are a wide array of microscopy techniques for studying cellular dynamics and function, from cheap and simple to use to costly and highly advanced techniques available. These include dark field, phase contrast, polarized microscope, and differential contrast microscope are called transmitted light microscopy and a variety of fluorescence microscopy techniques (Stephens and Allan, 2003; Culley et al., 2018). Nowadays, no live cell microscopy technique is suitable for all possible investigations (Jensen, 2013). The primary factors for the type of microscope to be used are the study's objectives, the researcher's expertise, interest, and financial viability, as well as the specimen's thickness, vitality, and sensitivity to detection (Stephens and Allan, 2003; Sanderson et al., 2014).

Although the various types of microscopy techniques are described in the literature, most studies have suggested a few/single types of microscopies. Compiling the common live cell microscopy techniques fragmented in different kinds of literature is very important to access all the techniques easily and comprehensively (Stephens and Allan, 2003). Transmitted light microscopy techniques are easy to use; however, they cannot detect subcellular morphology, especially

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in thick tissue samples. Fluorescent microscopy can reveal subcellular structures, including in thick tissue samples (Schmolze et al., 2011). However, diffraction only allows the convectional fluorescence microscope's resolution to reach roughly 200-300 nm (Culley et al., 2018). Conventional light microscopy's resolution can be improved by developing super-resolution techniques (Stephens and Allan, 2003). These methods break the diffraction limit, enabling fluorescence imaging with up to ten times better resolutions than traditional methods (Culley et al., 2018). This review aimed to describe the common live cell microscopy techniques and briefly explain their principles and applications.

DARKFIELD MICROSCOPY

In light microscopy, the objective lens collects two types of rays, namely diffracted rays and non-diffracted/undeviated rays. In bright field microscopy, the non-diffracted rays, also called zero-order rays, are essentially collected to form a bright background. These rays pass through the tissue but do not interact with the sample; therefore, they do not contain any information about the sample. In dark field conditions, the specimen is illuminated from the side such that those zero order/background/non-diffracted light rays are not collected by the objective lens. Therefore, the sample appears bright on dark background. To achieve this condition, dark field microscopy is equipped with a black disk or special dark-field aperture in the condenser (Murphy and Davidson, 2012).

Darkfield microscopy is used for many biological applications, including radioactive in situ hybridizations of mRNA expression, microbial detection, and diagnosis in clinical microbiology (Macnab, 1976; Chen et al., 2012).

PHASE CONTRAST MICROSCOPY

Biological specimens are usually transparent and do not absorb light but instead diffract it and cause phase shift. These specimens are called phase objects, meaning they can cause a phase shift when light passes through them; however, humans' eyes cannot detect these phase differences. Therefore, we need an instrument that converts phase shifts into amplitude differences. This activity is undertaken by a phase contrast microscope. For this purpose, a phase contrast microscope is equipped with two critical components named condenser annulus/phase annulus (a black plate positioned in front of the condenser) and a phase plate positioned at the objective lens. Phase contrast microscopy is widely used mainly for big structures (nuclei and other organelles) (Sanderson, 2001; Aryal, 2022). In addition, phase images are also often used as anatomical references for fluorescence signals (Sahu et al., 2006).

DIFFERENTIAL INTERFERENCE CONTRAST

Differential interference contrast (DIC) is one of the interference microscopy techniques (including phase contrast, and polarized microscopy) that generates contrast based on wave interference. It is important to visualize unstained, live, transparent cells. This method forms an image that has an impression of three dimensions. The DIC microscopy requires a polarized light source, two polarizers, and two special Wollaston prisms. When polarized light passes through the prism, which is called Wollaston prism 1, it will be divided into two components, pass through the sample, and will later be recombined at the second Wollaston prism. Superimposition of these two light components later by the analyzer results in images having contrast (Zernike, 1942; Murphy and Davidson, 2012). Differential interference contrast helps research live cells. In live, unstained tissue, it is incredibly helpful for resolving individual cells and cellular organelles. Slices of the brain, cell culture, and even whole organisms (such as embryos) can all be studied with DIC microscopy (Ziv and Schiller, 2007).

FLUORESCENCE MICROSCOPY

Transmitted light microscopy uses focused transmitted light that goes through a condenser to achieve a very high illumination on the specimen. In fluorescence microscopy, fluorescent dyes are used to label cells or molecules, and the light is reflected rather than transmitted to capture the images (Walker-DanielsandFaklaris, 2012). These molecules are capable of absorbance and emission of a specific range of wavelengths (Lichtman and Conchello, 2005). Fluorescence microscopy has a wide range of information-conveying "dimensions" because the light's spectra can be separated. This method might be used to carry out many simultaneous immune fluorescent labelings on a single slice. At least three colocalized antigens can be recognized simultaneously and individually if immunofluorescence is utilized. When using standard light microscopy, a reasonable resolution of no more than three to four colors, including any necessary counterstains, is possible (such as hematoxylin). However, colocalized antigens are difficult to separate in transmitted light microscopy (Schmolze et al., 2011).

Fluorescence microscopy became one of the most widely used and preferred modalities for live cell imaging (Wang and Lai, 2021). This is due to its very high contrast, sensitivity, specificity, and selectivity. Fluorescence microscopy provides a wide array of possible live cell studies, including motility, protein location, associations, and other phenomena such as ion transport and metabolism (Ettinger and Wittmann, 2014). There is a possibility of using different fluorescent dyes together to stain different components of the cell so that they can be detected simultaneously. This is rather barely possible with conventional transmitted light microscopy. Fluorescence microscopy has shown a

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great improvement over the last decade, which provides a wide array of research possibilities in live cell imaging (Fritzky and Lagunoff, 2013). This advancement is associated with increased use of fluorescent proteins, availability of numerous new fluorophores, development of different types of fluorescence microscopy techniques, including basic confocal microscope, and multi-photon microscopy for thick samples and better image contrast, and breaking of the resolution limit by the development of super-resolution microscopy (Shaner et al., 2005; Suzuki et al., 2007).

The fluorophore absorbs light, which causes its electrons to be stimulated from their ground state (lowest energy level) to an excited state (higher energy level). Since some energy may be lost as heat or other forms of energy during the electrons' descent to the ground state, they emit light with a longer wavelength when they return to their lowest energy state. The emitted light is seen as fluorescence. The phenomenon is described by Jablonski's diagram (Coling and Kachar, 1997; Jin and Riedel-Kruse, 2018). The difference between the excitation and emission maximum of a fluorophore is called the Stokes shift. Each fluorophore has a property of absorption of a specific wavelength of light best (or better than other wavelengths of light) and a characteristic emission of a specific wavelength. This property of fluorophores is called absorption and emission spectra. As a result, the peak wavelength of excitation and emission is the same for all fluorophores. Individual fluorophores' variations in excitation and emission spectra can be used to distinguish distinct targets within the same sample (Lichtman and Conchello, 2005).

Specific parts of biological samples can be labeled using fluorophores or in some cases, fluorophores can be found naturally in the sample of interest (Lichtman and Conchello, 2005). Fluorophores can also be introduced into organisms through genetic modification to the organism's DNA to encode fluorescent molecules (Chudakov et al., 2010; Thorn, 2017). An alternative method is tagging antibodies with small molecule fluorophores, such as Texas Red, and then introducing them into the specimen (Lichtman and Conchello, 2005).

WIDEFIELD FLUORESCENCE MICROSCOPY

Wide-field fluorescence microscopy (WFFM) is an imaging technique where the whole specimen is shown with light of a specific spectrum in which the resulting image is either viewed by the observer or captured with a camera (Sanderson et al., 2014; Wilson, 2017). The fundamental components of WFFM are the excitation light source, beam splitter/mirror, dichroic and emission filters, the camera, and the objective lens. The excitation of the fluorophore by the polychromatic light source results in an emission of a longer wavelength of light that can be visualized through an eyepiece or via a digital camera. Widefield fluorescence microscopy has three filters that have different purposes. The excitation filter allows only the light spectrum passage that best excites the fluorophore. The dichroic mirror reflects the excitation light to the specimen and permits the passage of the emitted light from the specimen to the emission filter. On the other hand, it ensures that only the wavelength emitted from the fluorophore in the sample pass through (Jin and Riedel-Kruse, 2018). TheWFFM detects the resulting image via multi-point detector or digital camera (Lichtman and Conchello, 2005).

Wide-field fluorescence microscopy is sensitive, affordable, and versatile for live-cell imaging that can generate beautiful high-resolution images 20. It is simple and fast because all specimen parts are illuminated simultaneously, allowing for easier and faster imaging with a camera. However, as wide areas of the sample are illuminated, optical diffraction and out-of-focus lights can result in blurry images (with low contrast and spatial resolution, Lichtman and Conchello, 2005; Sanderson et al., 2014). This can be improved using deconvolution, an image restoration technique that uses different algorithms to send back the out-focus light to its original position and provide a high-contrast image (Swedlow and Platani, 2002).

Wide-field fluorescence microscopy is a significant technique for the observation and identification of cells, cellular components, and localization of the protein (Scientifica, 2022). It is very crucial to examine thin specimens like cells of the monolayer (Wang and Lai, 2021). Wide-field fluorescence microscopy can produce an image with better resolution compared to conventional transmitted microscopy (Vangindertael et al., 2018). It is an appropriate tool for long-standing and comparatively swift quantitative time-lapse imaging. Using this technique, it is possible to visualize the amount and localization of specific fluorescent molecules in a very short period (Scientifica, 2022). For instance, cellular processes that take place within cell-like neuronal signaling can be quantified in real-time (Scientifica, 2022). Other advantages of WFFM include its relatively cheaper price compared to other advanced techniques such as confocal laser-scanning microscopy (CLSM), its high resolution in the XY dimension and very fast temporal resolution, and it relatively requires the least amount of excitation light (Coling and Kachar, 1997; Vangindertael et al., 2018).

CONFOCAL LASER-SCANNING MICROSCOPY

Confocal laser-scanning microscopy (CLSM) is a type of microscopy characterized by a pinhole opening that serves as a barrier to light originating from other focal planes in the sample to get rid of out-of-focus light from the image, which leads to better contrast with low noise and high resolution (Webb et al., 2004; Cole, 2014; Sanderson et al., 2014). A laser is used in place of a lamp in all CLSM systems, sensitive photomultiplier tube detectors (PMTs) are used

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to measure the intensity of light emitted, and computers are used to operate the scanning mirrors and assist in image collection and display (Ulrich, 2015).

In CLSM, a pinhole aperture on a confocal plane with a scanning point on the specimen and a second pinhole aperture in front of the detector allows consistent light, which is an excitation source, from the laser system to pass through. The secondary fluorescence emitted from points on the specimen (in the same focal plane) passes through the back side of the dichromatic mirror and is focused as a confocal point at the detector pinhole opening while the laser is reflected by a dichromatic mirror and scanned across the specimen in a defined focal plane. Out-of-focus light rays are the significant amounts of fluorescence emission that do not confocal with the pinhole and occur at sites above and below the objective focal plane (Hashimoto et al., 2007). As a result, the photomultiplier detects only fluorescence originating from the in-focus sample plane (Ulrich, 2015).

It is an essential technique with a broad range of applications in biomedical research (López-Macay et al., 2016). Confocal laser-scanning microscopy is required when working with thick specimens, such as tissue slices and small organisms/live animals like Drosophila or zebrafish, to eliminate out-of-focus light. Due to the ability to produce serially thin optical sections (Webb et al., 2004; Cole, 2014). Confocal microscopy is particularly useful for acquiring and analyzing images from thick specimens, such as tissue samples containing structures like cells, and for observing subcellular structures, which are either autofluorescent or have been targeted with fluorescent molecules (López-Macay et al., 2016). Because of how much light is rejected by the pinholes and the low quantum efficiency of the photomultipliers used to create the image, the photon dose needed for imaging in CLSM is significant (Cole, 2014). Nevertheless, CLSM scans point by point with the so-called raster imaging, which is a very slow process. Slowness in microscopy may increase the vulnerability to Photobleaching (Webb et al., 2004).

MULTI-PHOTON CONFOCAL LASER-SCANNING MICROSCOPY

Multi-photon confocal laser-scanning microscopy (MP-CLSM) is equipped with a spinning Nipkow disk comprising an array of pinholes and microlenses and uses arc-discharge lamps for illumination instead of lasers and multiple-beam microscopes to readily capture images with an array detector (Ulrich, 2015). A single fluorophore molecule's ability to simultaneously absorb two or more low-energy photons of light is the foundation of multi-photon confocal laser-scanning imaging (Cole, 2014).

Multi-photon confocal laser-scanning microscopy is a compelling technique for examining thick tissues and uses more than one longer wavelength of light (infrared and near-infrared) wave instead of one with high energy/short wavelength (Andresen et al., 2009). Hence, the out-of-focus light produced is quite faint and does not excite anything to an appreciable level, the only area where excitation comes from is at the focal point. Therefore, light is obtained only from the in-focus spot, as a result, and there is no requirement for a pinhole. As a result, MP-CLSM is more efficient for collecting light as it does not require a pinhole. High light penetration into the living body is obtained while infrared and near-infrared rays are used. This makes MP-CLSM a preferable technique for visualization and examination of thick samples (usually for thicker than 100 µm samples) (Mülter, 2019; Schneckenburger and Richter, 2021). Confocal laser-scanning microscopy/confocal microscopy can be utilized for samples up to 100 micrometers thick, but for thicker specimens, it is not possible to eliminate all of the focus light via the pinhole in CLSM (Dunn and Young, 2006; Schneckenburger and Richter, 2021).

Having a lower photobleaching effect is another advantage of MP-CLSM over CLSM since MP-CLSM uses longer wavelengths. On the contrary, the image of MP-CLSM has a lower resolution, compared to CLSM because the resolution is the function of the excitation light wavelength (Rocheleau and Piston, 2003). In MP-CLSM, a single laser excitation is used for more than one fluorophore instead of multiple lasers for different fluorophores. This results in limited chromatic aberrations. Hence, MP-CLSM is ideal for co-localization studies or co-dynamic measurements such as Förster resonance energy transfer (FRET), or two-color image-correlation microscopy (Piston, 2006; Kawano et al., 2008). Multi-photon confocal laser-scanning microscopy can also be used for longitudinal studies of cortical structures of the mouse brain at high optical resolution (Grutzendler et al., 2011).

SPINNING-DISK CONFOCAL MICROSCOPY

Unlike confocal microscopy, spinning-disk confocal microscopy (SD-CM) possesses many pinholes. Hence, spinning-disk confocal microscopy is equipped with multi-point detectors, such as complementary metal oxide semiconductors and charge-coupled device cameras (Bai et al., 2020). As mentioned before, cameras have higher quantum efficiency than single-point detectors such as photomultipliers. Therefore, SD-CM is a preferred microscopy method for dim samples. In addition, the architecture of multiple pinholes provides SD-CM with the associated great speed. Hence SD-CM is better than confocal microscopy in time-lapse live cell studies because it is associated with shorter exposure time and minimization of photobleaching. Nevertheless, confocal microscopy is still a method of choice for thick samples; SD-CM and WFFM are rather preferred for thin samples (Schneckenburger and Richter, 2021). With spinning disc confocal microscopy, there is a slight loss of confocality due to the possibility of some out-of-focus light

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leaking through many pinholes; however, this is made up for by a superior signal-to-noise ratio and a reduced risk of photobleaching (Stehbens et al., 2012).

STIMULATED EMISSION DEPLETION MICROSCOPY

The resolution of a light microscope is limited by diffraction to about 200-300 nm (Culley et al., 2018). Therefore, objects residing at a closer distance than 200 nm, cannot be distinguished. This is why electron microscopy was invented, and its higher spatial resolution has allowed us to make great discoveries in life sciences. However, it is also evident that it is impossible to use an electron microscope to examine a cell in three dimensions, especially inside a living cell or living tissue. Therefore, using focus visible light is required. Hence, an invention of the light microscope that would overcome the diffraction barrier and provides an image with a very good spatial resolution comparable to the electron microscope would be very important (Hell and Wichmann, 1994; Meyer et al., 2008).

The primary component of a light microscope, the objective lens, is used to concentrate light onto a single point. However, because light travels as a wave, the lens is unable to focus all of the light into a single spot. This will cause the light to spread out and create a blob that is at least 200 nm across the focal plane. This blob is termed a point spread function. All the features falling within this spot are flooded at the same time with light. In fluorescence microscopy, excitation light is utilized, and for the same reason explained above, all the features will give of signal and be collected by the lens, and impossible to tell these features apart. To separate two objects by a light microscope, they have to be apart by the distance "D", which can be calculated by dividing the wavelength of light twice by the numerical aperture of the objective lens as given in the following Formula 1 (Bagnell, 2012; Diaspro and Bianchini, 2020). Where D is the distance between the objects, Lambda is the wavelength of light, and NA is the numerical aperture D = lambda / 2 NA (Formula 1).

The least possible value accounts for at least about 200 nm in XY and up to 500 nm in the Z dimension. In stimulated emission depletion (STED) microscopy, it is not only one beam for exciting molecules that is focused into the 200 nm range used, but also a beam of light that has a typical shaped-donut is also used (Culley et al., 2018). As it is not required to shut off all molecules, the STED beam has been shaped into a donut pattern. It is needed to keep an area where the molecules are still capable of emitting. Only a portion of the molecules is permitted to emit, although all of them are colored by excitation light, and the remainder is silent. This is achieved by using photons that do not have an energy that is high enough to excite molecules. If the photon energy fits the fluorescence data, the energy between the fluorescent state and the ground state, those photons are capable of sending molecules back down to the ground state instantly by taking away the majority of the energy in the red-shifted beam. The beam here is red-shifted because it has lower photon energy, and its role is simply to silence the molecules. The right wavelength should be utilized to silence the molecules. However, there is a need to make sure that there are enough red photons in the red-shifted beam. This is because if there are enough red photons, it is possible to be sure that once a molecule gets excited, there is always a red photon out there that will instantly kick the molecule down to the ground state (Hell and Wichmann, 1994; Meyer et al., 2008; Wildanger et al., 2009).

LOOP MICROSCOPE

A Loop microscope is a remarkable method that enables us to observe and quantify surface structure with unprecedented precision and detail (West and Eaton, 2010). With its ability to manipulate objects with nanometer-scale features, an image in a vacuum, air, or liquids with sub-nanometer resolution, and measure forces with greater than pico-Newton resolution, the loop microscope has become a beneficial instrument in a wide range of disciplines (Abramovitch et al., 2007). It can be used to photograph samples that are compliant, such as biologically important materials. This ability has been utilized to examine mechanical dynamics and characteristics at individual cells' level down to individual molecules. The *in-situ* analysis of drug-induced alterations in cell shape, membrane stability, and receptor contact forces, as well as the investigation of cell motility, are a few effective uses at the cellular level (Abramovitch et al., 2007; Hessenauer et al., 2021). The activity of RNA polymerase, the motion of molecular motors, including proton-powered turbines and myosin V, the transcription process, and the structure of a wide range of viruses have all been studied using single molecules (West and Eaton, 2010). To examine the mechanical characteristics of biological structures and the forces of molecular interactions, this microscope has also been extensively employed as a force transducer (Abramovitch et al., 2007).

The loop microscope is a helpful actuator for nanotechnology and can manipulate material as well. Employing customized tips makes it possible to apply a range of forces, including contact, magnetic, thermal, and electrical forces. It has been applied to nano assembly, nano manipulation, and lithography (West and Eaton, 2010). The loop microscope will remain a crucial tool in the researcher's toolkit due to the ongoing interest in understanding materials and biological systems at the nanoscale and the promise of nanotechnology (Abramovitch et al., 2007; Hessenauer et al., 2021). Unlike most microscopes, the loop microscope performs differently, and the probe physically interacts with the sample (West and Eaton, 2010). The loop microscope loop starts with a sample to image. The sample is usually on a raster-scanned

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surface back and forth. When a cantilever's sharp tip is brought in close contact with a surface, the cantilever deflects due to the interaction between the tip and the surface. The resulting deflection is measured by reflecting a laser beam off the back of the cantilever onto an optical detector. An optical detector's deflection signal is compared to a nominal deflection value that represents the imaging force, and the difference is reduced by employing a feedback controller. The surface profile is often estimated from the control signal itself (Meyer, 1992; Abramovitch et al., 2007; West and Eaton, 2010).

CONCLUSION

Different types of microscopy techniques can be used to study live cells and tissues. All live cell microscopy techniques discussed have their pros and cons. Decisions on the choice of the microscopy technique should be made based on the objective of the research and the capability of the technique to provide the required information without damaging the tissue. For this purpose, optical light paths should be optimized for sensitivity, speed, and resolution based on the objective of the research. Hence, a thorough understanding of the technique and application of the various live-cell microscopy methods is paramount.

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Authors' contribution

Tewodros Abere Mekuria drafted the manuscript. Mebrie Zemene Kinde edited the draft of the manuscript, and prepared it for publication. The authors read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

All authors have reviewed their work for ethical problems, such as plagiarism, consent to publish, misconduct, data fabrication and/or falsification, duplicate publishing and/or submission, and redundancy.

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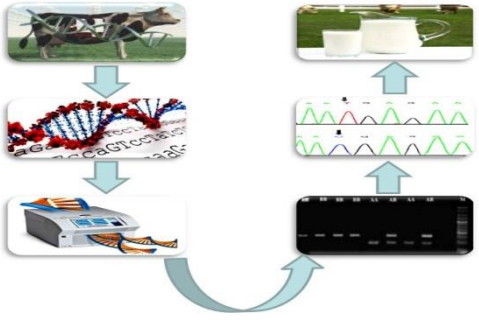

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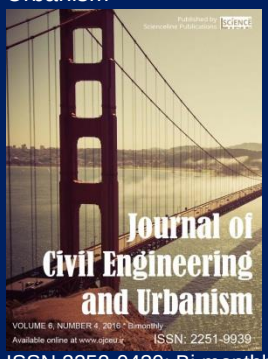

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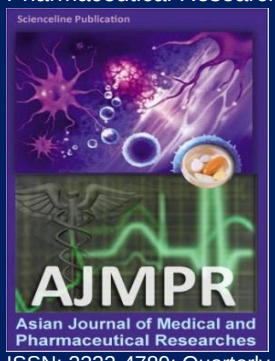

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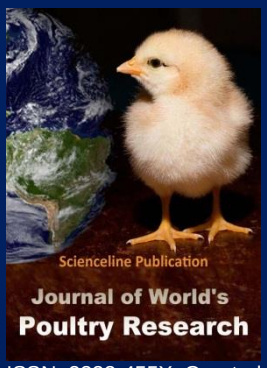

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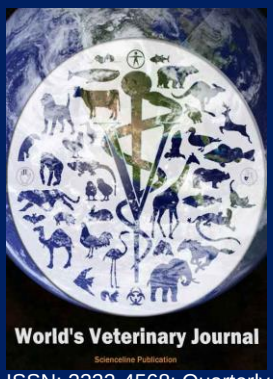

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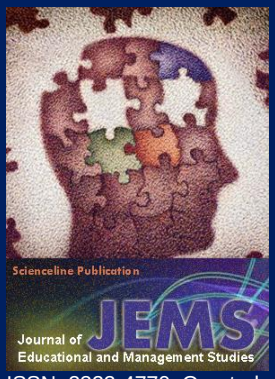

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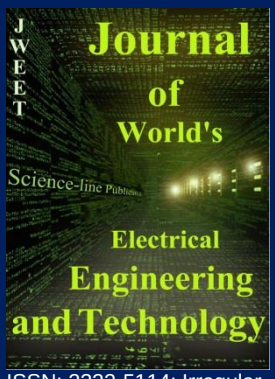

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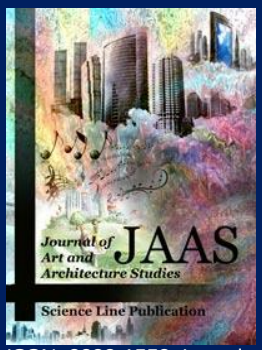

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