2025, Scienceline Publication
World's Veterinary Journal
World Vet J, 15(2): 229-237.
ISSN 2322-4568

DOI: https://dx.doi.org/10.54203/scil.2025.wvj25
PII: S232245682500025-15

GATA4 Gene Expression Pattern and Stomach Histogenesis in White New Zealand Rabbit Fetus (*Oryctolagus cuniculus*)

Doaa S. Nouh[1]*, Maha S. Kilany[2], Nagwa Ibrahim[3], Eman H. Elsheikh[4], and Esraa Elsheikh[1]

[1]*Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, 44511*
[2]*Department of Histology and Cytology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, 44511*
[3]*Department of Husbandry and Animal Wealth Development, Faculty of Veterinary Medicine, University of Sadat City, Sadat City, Egypt, 32897*
[4] *Department of Comparative Anatomy, Faculty of Science, Zagazig University, Zagazig, Egypt, 44511*

*Corresponding author's Email: doaanooh14@gmail.com

ORIGINAL ARTICLE
Received: March 17, 2025
Revised: April 21, 2025
Accepted: May 24, 2025
Published: June 25, 2025

ABSTRACT

There is a scarcity of published data regarding embryonic development in rabbits. The current study was conducted to evaluate the development of the stomach in rabbit fetuses and the expression of the transcription factor *GATA4* during different stages of development. This study was conducted on 40 rabbit fetuses for morphology, histology, and *GATA4* expression. The rabbits used were seemingly healthy white New Zealand pregnant females (aged 1.5- 3 years and weighing 2- 4.5 kg). The morphology and histology of rabbit fetuses' stomachs (22 fetuses) were examined using a light microscope on days 10, 12, 13, 14, 16, 18, 20, 22, and 26 of the rabbit's gestation period. The expression of *GATA4* in rabbit fetuses (18 fetuses) on developmental days (20, 22, and 26) was investigated using quantitative real-time PCR (qRT-PCR). The results indicated that the initial stomach appeared as a fusiform widening in the posterior part of the foregut on day 10. The mucous membrane was significantly thickened, and the formation of the gastric gland commenced on day 18. On day 22, the lining epithelium transformed into the pseudostratified epithelium, and the mucosal folds exhibited more clarity. On day 26, the lining epithelium of the stomach had changed into a simple columnar epithelium. Additionally, the mucosal folds and gastric pits were fully developed in all stomach areas. The stomach glands displayed exceptional transparency and were fully developed. Analysis of qRT-PCR data showed a significant increase in *GATA4* mRNA expression in the embryonic stomach on gestational days 20, 22, and 26 as the pregnancy progressed. In conclusion, this study comprehensively described the morphological and histological development of the rabbit stomach. It demonstrated a substantial upregulation of *GATA4* mRNA expression as gestation advanced, indicating its crucial role in stomach development.

Keywords: Development, Fetus, *GATA4*, Primitive stomach, Rabbit

INTRODUCTION

Rabbits have proven to be highly advantageous models in various fields of biological study, such as embryology, toxicity, and pathology. The internal organs are the main subject of research that requires a thorough understanding of their exact location, growth, structure, and tissue composition (Burkholder et al., 2012).

The stomach is a vital organ responsible for various crucial processes, such as food digestion, immunological defense, and maintenance of metabolic homeostasis (Willet and Mills, 2016; McCracken and Wells, 2017). The monolocular stomach of rabbits comprises three sections, including the cardiac, fundic, and pyloric regions. The latter three regions contain distinct types of glands (Ranjan and Das, 2018).

During early embryonic development, the digestive tract initially forms a simple gut tube without any distinct features. As development continues, it becomes regionalized into three sections, including the foregut, midgut, and hindgut, as described by Esrefoglu et al. (2017). The primitive stomach first develops as a fusiform dilation in the posterior part of the foregut (Sadler, 2018).

The stomach experiences significant alterations in its size, location, rotation, and shape during morphogenesis (Macarulla-Sanz et al., 1996). The origin of this commences from two germ layers, namely the endoderm, responsible for the development of the mature epithelium lining, and the mesoderm, which contributes to the surrounding connective tissue and smooth muscle (Willet and Mills, 2016). The reciprocal interactions between epithelial and mesenchymal cells play a crucial role in promoting the growth of the stomach during fetal development, as well as its ongoing regeneration and differentiation in adult life (McCracken and Wells, 2017; Esrefoglu et al., 2017) in humans. Sultan et al. (2018) stated that in rabbits, the stomach primordium emerges on day 10 of gestation as a dilation of the foregut. The significance of evaluating fetal gastric development and its relevance to treating human diseases, research has shown that understanding the molecular and morphological changes during stomach formation can provide insights into congenital

To cite this paper: Nouh DS, Kilany MS, Ibrahim N, Elsheikh EH, and Elsheikh E (2025). *GATA4* Gene Expression Pattern and Stomach Histogenesis in White New Zealand Rabbit Fetus (*Oryctolagus cuniculus*). *World Vet. J.*, 15(2): 229-237. DOI: https://dx.doi.org/10.54203/scil.2025.wvj25

gastrointestinal disorders. Studies on animal models, such as rabbits, help identify key regulatory genes and pathways involved in gastric development, which may aid in addressing conditions like pyloric stenosis and gastric atresia. Furthermore, advancements in fetal stomach research contribute to regenerative medicine by paving the way for bioengineered stomach tissues and novel therapeutic strategies for human gastric disorders (Sadler, 2018).

Sultan et al. (2018) found that the lining epithelium of the stomach in 10-day-old rabbit embryos is stratified. However, by day 27, it transforms into a simple columnar epithelium throughout all parts of the stomach, except for the area around the cardia, where it remains stratified squamous epithelium. According to Soliman et al. (2020), the gastric epithelium shifts from a stratified to a simple columnar structure on day 21 of gestation in white New Zealand rabbits. This transformation is marked by the formation of distinct stomach regions, including the cardia, fundus, body, and pylorus, which become distinguishable. By day 23 of gestation in rabbits, the epithelium develops distinct folds, while the gastric mucosa is composed of the lamina epithelialis, lamina propria, and muscularis mucosa (Hayward, 1967; Soliman et al., 2020).

The overlapping patterns of gene expression are very important because they control endoderm differentiation at the molecular level (Hyttel, 2010). *GATA4* is expressed throughout the definitive endoderm in the foregut during the process of endodermal regionalization. Specific expression of *GATA4* occurs at the boundary between the future forestomach and glandular stomach, where it plays a distinct role in specifying the development of the glandular stomach. Another unique job of *GATA4* is to help the definitive endoderm turn into the gastric columnar glandular epithelium (Jacobsen et al., 2002; Thompson et al., 2018).

As far as the authors know, there is a significant lack of research on the normal development of the stomach in fetal rabbits. Hence, the primary goals of this study were to examine the development of the stomach in rabbit embryos and fetuses, spanning from the initial embryonic phase to the last fetal stage, through the utilization of light microscopy. Furthermore, the present study aimed to highlight or address this lack of data by investigating *GATA4* expression in certain developmental stages of rabbit fetuses, utilizing qRT-PCR.

MATERIALS AND METHODS

Ethical approval

All techniques were performed following the guidelines of Zagazig University's Institutional Animal Care and Use Committee (ZU-IACU/2/F/216/2024), Egypt.

Sample's collection

This study was conducted on a sample of 40 healthy white New Zealand rabbit embryos and fetuses, on days 10, 12, 13, 14, 16, 18, 20, 22, and 26 of fetal life. The rabbits used in this study were sourced from seemingly healthy white New Zealand pregnant females (aged 1.5- 3 years and weighing 2-4.5 kg). The rabbits were sourced from the Research Farm of the Faculty of Veterinary Medicine at Zagazig University, Egypt, and rabbit farms in Al-Sharkia Governorate, Egypt. Pregnant rabbits have increased nutritional demands to support both their own metabolic needs and the growth of their offspring. Consequently, their diet should be supplemented with a higher quantity of fresh vegetables, and alfalfa hay should be available ad libitum to ensure adequate nutrient intake. They were housed in well-ventilated, galvanized wire battery cages within a controlled indoor environment to maintain hygiene and welfare standards. A gradual feeding regimen was implemented to minimize the risk of digestive disturbances. Fresh, clean water was supplied ad libitum throughout the day. Rabbit mating was conducted by housing a male and a female in the same cage, allowing for natural copulation. After cohabitation, the animal care staff observed the animals daily to identify signs of successful mating and determine the day zero of pregnancy. Routine monitoring involves behavioral assessments and physical examinations to detect early pregnancy indicators.

Histological examination

All surgical procedures were conducted under anesthesia to decrease suffering in rabbits. The pregnant female rabbits were anesthetized via intramuscular injection of 35 mg/kg ketamine hydrochloride (HCl) (KETALAR, 100 mg/ml, Pfizer, New York) and 5 mg/kg xylazine (Xylaject, 20 mg/ml, ADWIA, Egypt), both drugs were administered together in one injection. For a rabbit weighing 3 kg, the dose will be 1.8 ml from both drugs, so the single injection differs according to the rabbits' weight following the protocol outlined by Lipman et al. (2008) and Enoka (2013). Subsequently, the does were euthanized by exsanguination after being rendered fully unconscious (Close et al., 1997), and the embryos were extracted after evisceration. The freshly obtained embryos were fixed in either 10% neutral buffered formalin for 24 hours or Bouin’s fluid for 8 to 24 hours before being transferred to a 70% ethyl alcohol solution. The fixed specimens underwent dehydration through a graded series of alcohol, followed by clearing, paraffin

embedding, and serial sectioning at a thickness of 3-5 µm using a Rotary Manual Microtome M380 (Germany). The sections were then stained with Harris hematoxylin and eosin. The histological technique was performed according to Berg et al. (2011). The sections were examined microscopically (OPTICA Microscope with C-B5 digital camera, OPTICA Italy). The mean crown -vertebra- rump lengths (CVR) for all specimens analyzed in this study are presented in Table 1.

Table 1. The average CVR lengths of embryos and fetuses in relation to their ages according to the gestational period of the rabbit (average 30 days).

Age of the embryos and fetuses	Average CVR length in cm
Day10 embryos	±0.52 cm
Day 12 embryos	±0.9 cm
Day 13 embryos	±1.1 cm
Day 14 embryos	±1.3 cm
Day 16 embryos	±2 cm
Day 18 fetuses	±2.5 cm
Day 20 fetuses	±3.2 cm
Day 22 fetuses	±4.5 cm
Day 26 fetuses	±5.8 cm

Gene expression (qRT-PCR)

The qRT-PCR was performed at the Molecular Biology Laboratory Centre, Faculty of Veterinary Medicine, Benha University, Egypt. Nine samples of the stomach of rabbit fetuses were gathered, three from each gestation day 20, 22, and 26. The samples were stored at -80°C until RNA extraction.

***RNA* purification and reverse transcription**

Total RNA was extracted from stomach samples using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. After tissue disruption and homogenization, ethanol was added to the lysate and transferred to the RNeasy Mini spin column. High-quality RNA was eluted in RNAse-free water after impurities had been effectively washed from the membrane. Namedrops (Denovix®, USA) absorbance was evaluated to determine the concentration and purity of RNA samples according to Pahlevan (2014).

According to TOPscriptTM cDNA Synthesis Kit (enzynomics, Korea) manufacturer's instructions, cDNA samples were reverse transcribed from isolated total RNA and preserved at -20 ^{o}C for further analysis.

Gene expression by qRT-PCR

The GATA4 gene was expressed at the level of mRNA in stomach tissues using a real-time PCR thermal cycler (Eppendorf, Germany). Primers of target and internal reference genes were designed and listed in Table 2. Quantitative RT-PCR was carried out using a SYBR Green qPCR master mix (TOPreal qPCR 2X PreMIX) according to the manufacturer's instructions. The cycle profile used for qRT-PCR was as follows: Holding at 94 °C for 10 minutes, followed by 40 cycles of 94 °C for 15 s, 64 °C for 30 s, and 72 °C for 30 s. After every PCR cycle, a melting curve analysis was conducted to verify the amplification's specificity. Duplicates of every sample were used (Yilmaz et al., 2012).

Normalization of the data for the *GATA4* gene was done in relation to the internal reference gene, *GAPDH*. The Ct values of *GAPDH* in each sample were subtracted from the gene of interest to achieve normalization of data. The value that was obtained by substracting the Ct value of the *GAPDH* gene from the Ct values of the GATA4 gene in each sample was called the ΔCt value. Using the comparative Ct method and $2^{\wedge-\Delta\Delta Ct}$ formula, the $^{\Delta\Delta Ct}$ corresponds to the difference between the $^{\Delta Ct}$ measured for 20, 22, or 26 days of gestation and the ΔCt of the calibrator. The relatively old change in the gene of interest was ascertained according to Livak and Schmittgen (2001).

Table 2. Primer sequences of the *GATA4* and *GAPDH* genes that were used for qRT-PCR analysis

Target genes	Accession no.	Sequence (5' to 3')	Product size by base pairs (bp)	Reference
GATA4	XM_002709438.4	**F**: 5′- CCGACACCCCAATCTTGTAGAC- 3` **R**: 5′- CAGGTAGTGTCCTGTCCCGT - 3`	3563 bp	Tremblay et al. (2018)
GAPDH	NM_001082253.1	**F**: 5′- GTCAAGGCTGAGAACGGGAA -3` **R**: 5′- CCAGCATCACCCCACTTGAT -3`	1282 bp	Ayyat et al. (2024)

Statistical analysis

GATA4 gene relative fold changes levels during different gestation periods (days 20, 22, and 26) were compared using a one-way ANOVA test followed by Tukey's post-hoc test, using SPSS software, version 25 (SPSS for Windows, V 25.0; SPSS Inc., Chicago, IL, USA) between the latter three groups for significance comparison. The results are shown as means ± standard deviation (S.D.), and the statistically significant level was defined as $p \leq 0.05$.

The nomenclature used in the current study was adopted as closely as possible to Nomina Anatomica Veterinaria (2017), Nomina Embryologica Veterinaria (2017), and Nomina Histologica Veterinaria (2017), which are known as standard veterinary anatomical, embryological, and histological nomenclature guidelines.

RESULTS

The histological findings revealed that on day 10, the primitive stomach developed into a fusiform widening in the posterior foregut. The foregut's endodermal cells were cuboidal in shape, with large, rounded vesicular nuclei that exhibited mitotic division and a pale cytoplasm. The epithelium lining the foregut gradually transformed into a more complex stratified structure, consisting of 3 to 5 layers. This stratified epithelium was enclosed by a delicate layer of mesenchymal tissue (Figure 1A, B). The surrounding mesenchymal tissue comprised undifferentiated connective tissue with fibroblasts, characterized by large, intensely stained nuclei. On day 12, the stomach continued its growth and differentiation from the foregut caudally to the abdominal cavity. The stomach was formed from a narrow lumen surrounded by stratified epithelium. The lining epithelium became more crowded and complex compared to that observed on day 10. It was composed of many layers of cells with oval and rounded vesicular nuclei. The epithelium was encircled by a dense layer of vascular mesenchymal connective tissue, with no distinct boundary between the two layers (Figure 1C, D). On day 13, the stomach enlarged, and its inner cavity became bigger. The lining epithelium became more distinct from the underlying mesenchymal connective tissue, but it remained stratified with fewer layers compared to day 12 (Figure 1E, F).

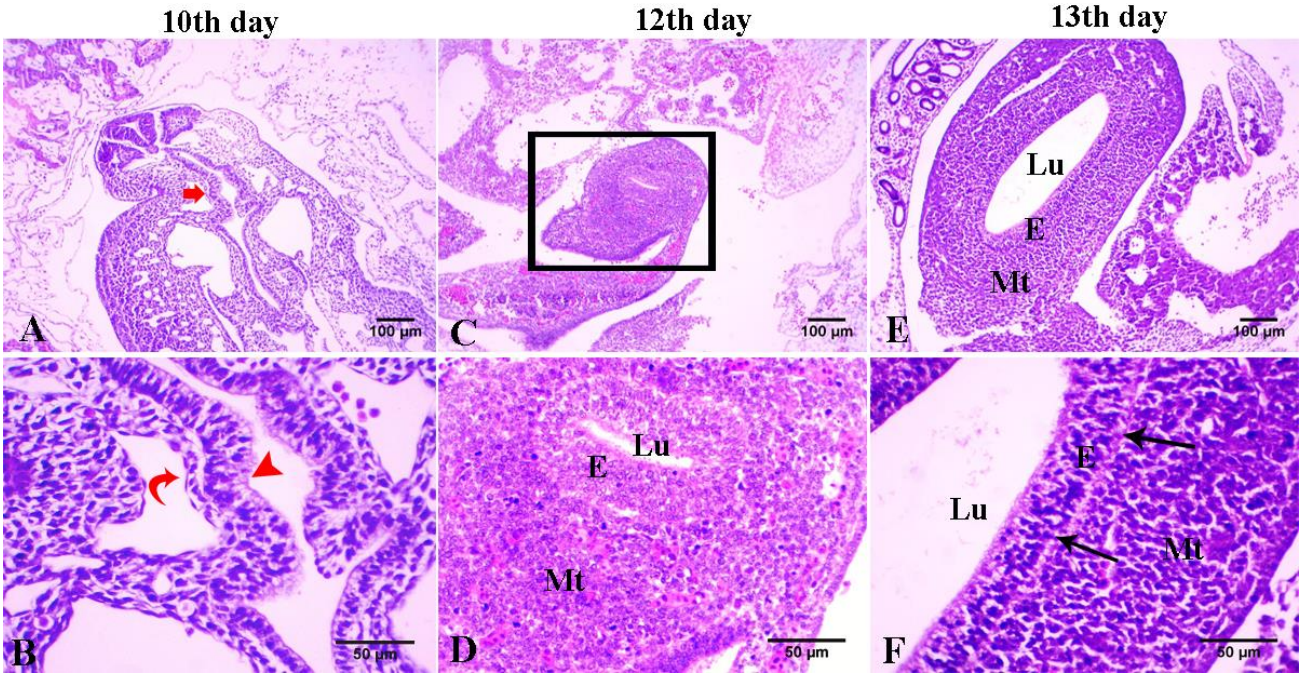


Figure 1. The prenatal histological changes in the rabbit stomach at days 10 (**A** and **B**), 12 (**C** and **D**), and 13 (**E** and **F**). Dilatation in the posterior part of the foregut (red thick arrow), stratified epithelium of the primitive stomach (red arrowhead), and a fine layer of mesenchymal tissue (red closed arrow). The stomach (Squared area), lumen (Lu), the lining epithelium (**E**), mesenchymal connective tissue (Mt), and the basement membrane (black long arrows). **H** and **E** Staining.

On day 14, the epithelium lining of the stomach was still stratified, and the mesenchymal connective tissue became more differentiated compared to day 13. However, the layers of the stomach wall were still not well-defined (Figure 2 A, B). As gestation progressed, the stomach wall became more specialized and distinct. By day 16, the layers of the stomach wall became more differentiated, and the tunica muscularis started to appear (Figure 2C, D). On day 18, the tunica mucosa was thick, and an alveolar gastric gland-like structure began to develop. The lining epithelium cells had oval apically located nuclei and basophilic cytoplasm (Figure 2E, F).

On day 20, the lining epithelium of the stomach remained stratified, and the gastric pits were still not visible. The tunica muscularis was still discontinuous, appearing in certain regions and missing in others (Figure 3A, B). On day 22, the lining epithelium transformed from stratified to pseudostratified epithelium. The mucosal folds were more distinct; the gastric pits began to emerge as invaginations in the epithelium, and the gastric gland began to appear. The lamina muscularis mucosa began to appear, and the tunica muscularis became well-developed in all regions (Figure 3C, D). On day 26, the lining epithelium transformed into a simple columnar epithelium, and the mucosal folds and gastric pits were well-developed in all regions of the stomach. The gastric glands exhibited a high degree of clarity and were fully grown (Figure 3E, F).

With the progression of the gestation period, qRT-PCR data analysis indicated a considerable upregulation in the mRNA expression level of *GATA4* in the embryonic stomach. During days 22 and 26 of gestation, respectively, there was a significant increase in the level of *GATA4* gene expression that was 1.27 and 1.89 times higher than it was during day 20 of gestation ($p < 0.5$) (Figure 4).

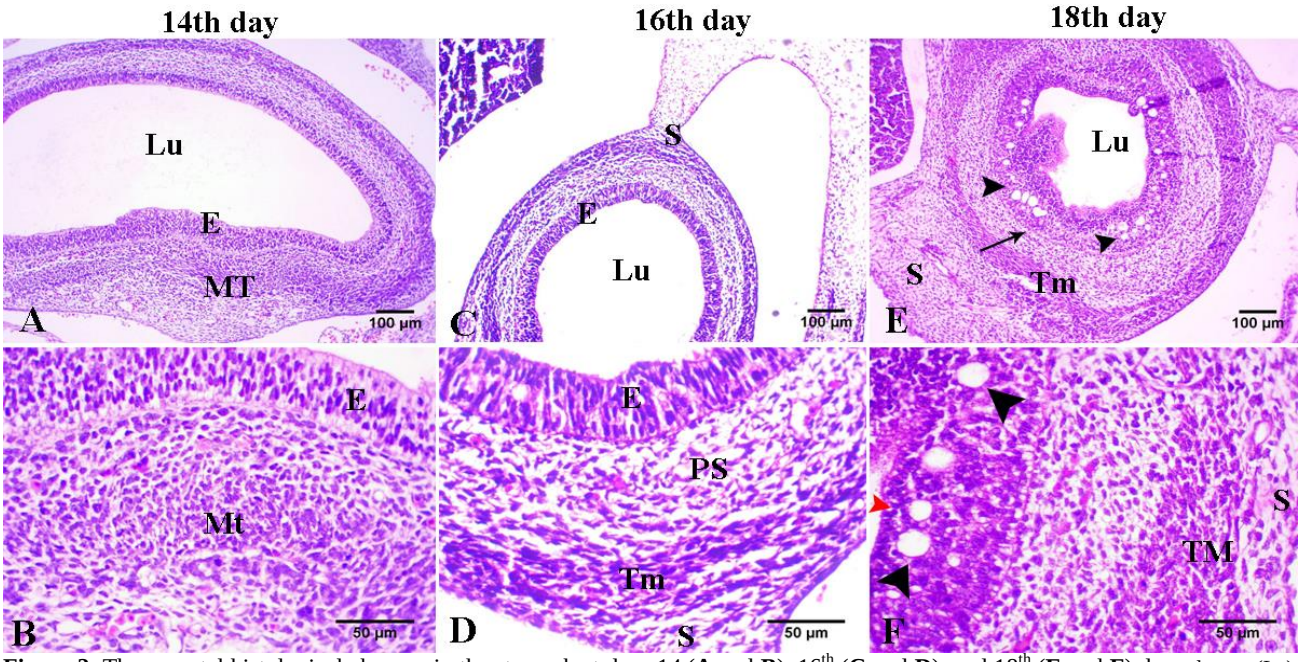


Figure 2. The prenatal histological changes in the stomach at days 14 (**A** and **B**), 16th (**C** and **D**), and 18th (**E** and **F**) days. lumen (Lu), the lining epithelium (E), mesenchymal connective tissue (Mt), serosa (S), propria submucosa (ps), and tunica muscularis (Tm). The beginning of the alveolar gastric gland (arrowheads) and the basement membrane (long arrows). **H** and **E** Staining.

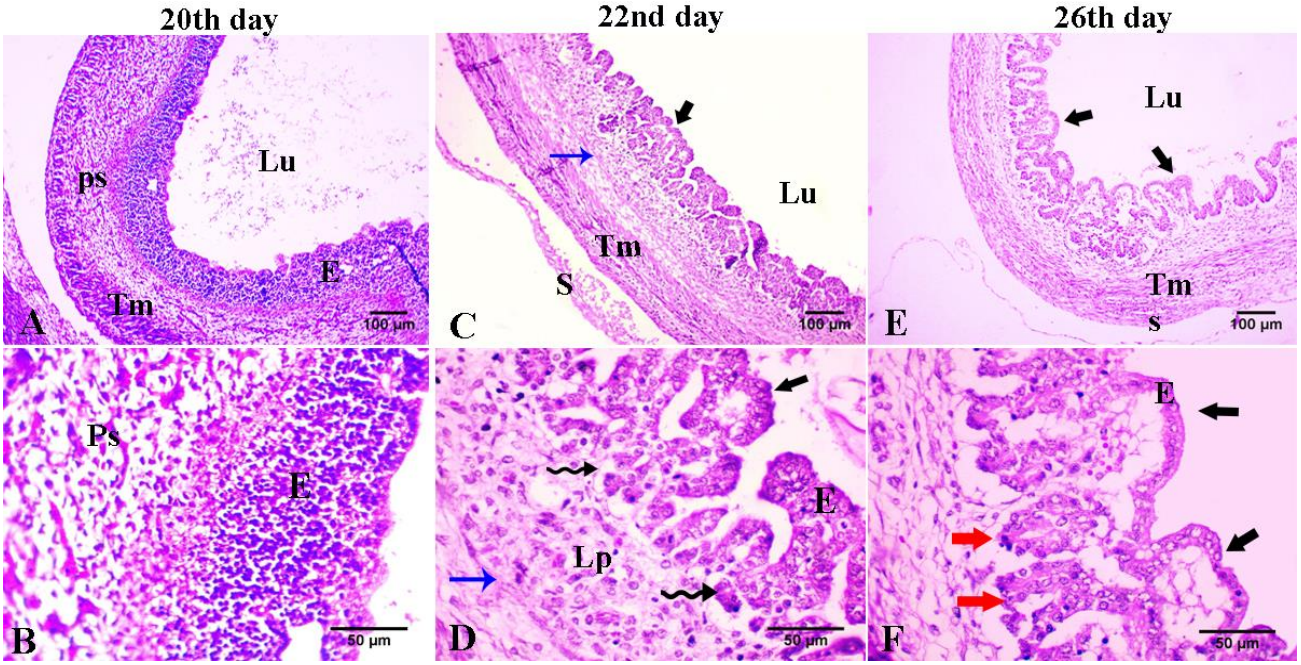
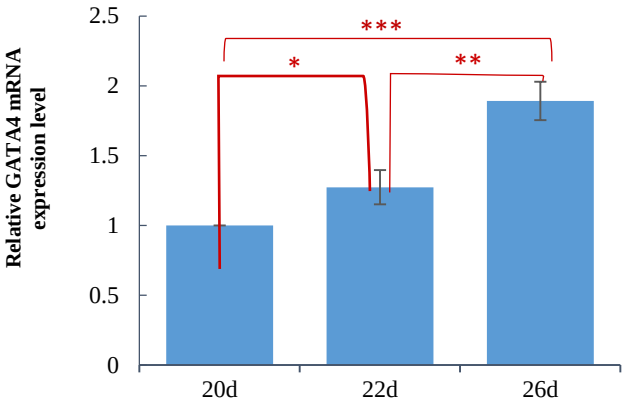


Figure 3. The prenatal histological changes in the stomach at days 20 (**A** and **B**), 22 (**C** and **D**), and 26 (**E** and **F**). Lumen (Lu), the lining epithelium (E), propria submucosa (ps), tunica muscularis (Tm), serosa (S), lamina propria (Lp), mucosal folds (black thick arrows), muscularis mucosa (blue arrow), beginning of the gastric gland formation (zigzag arrow), well developed gastric gland (red thick arrows). **H** and **E** Staining.



Group	Mean ± SD
Day 20	1 ± 0^{c}
Day 22	1.27 ± 0.122^{b}
Day 26	1.89 ± 0.137^{a}

Figure 4. Relative change in *GATA4* gene expression level in the stomachs of fetal rabbits during days 20, 22, and 26 of gestation. Data are presented as mean ± SD of 3 fetuses at each sampling time. * P = 0.045, **P = 0.001 and ***P < 0.001. Different superscript letters indicate significant differences (P < 0.05, Tukey's multiple comparison tests) among groups.

DISCUSSION

This study aimed to investigate the sequential phases of rabbit stomach development, starting from the initial embryonic stage and progressing through to the advanced fetal stage. The present investigation revealed that on day 10, the primitive stomach emerged as a fusiform expansion from the posterior foregut. The latter findings were reported in previous studies (Esrefoglu et al., 2017 in rats; McCracken and Wells, 2017; Sadler, 2018; Sultan et al., 2018). In mouse fetuses, the primitive stomach was visible at embryonic day 10.5 and got noticeably larger by day 11.5 (Li et al., 2014). In pig fetuses, Hyttel et al. (2010) added that the stomach originated as a spindle-shaped dilation of the caudal region of the foregut, becoming visible around the third week of gestation.

The current study indicated that the lining epithelium of the stomach was stratified, consisting of multiple layers. By day 26, it underwent a transformation into a simple columnar epithelium in all parts of the stomach. Sultan et al. (2018) made similar observations. Hayward (1967) reported that on day 18 of gestation, the rabbit stomach epithelium was composed of tall columnar cells. In addition, Soliman et al. (2020) presented that the rabbit stomach epithelium underwent a transition from a stratified to a simple columnar structure on day 21 of gestation.

In this study, the stomach pits were shown to develop as invaginations in the epithelium during day 22 of gestation. These invaginations subsequently developed into shallow primitive tubular or alveolar gastric glands. Yaman and Girgin (2001) and Soliman et al. (2020) reported similar observations in rabbits on day 21 of stomach development. Esrefoglu et al. (2017) observed similar findings in rat fetuses on day 15 of stomach development, while Fukamachi (1979) reported similar observations in mouse fetuses on day 15. Nilnophakoon (1984) indicated that in pig fetuses, the formation of gastric pits resulted from a depression in the gastric mucosa toward the basement membrane. Additionally, the elongated portion of the pit contributed to the development of gastric glands.

The present observations of the gastric glands, which exhibited significant development and took the form of branched tubular glands on day 26 of rabbit stomach development, were consistent with the findings reported by Sultan et al. (2018) and Soliman et al. (2020) in rabbit embryos.

According to the findings of the present study, on day 22 of rabbit development, the lamina muscularis mucosa started to emerge, and the tunica muscularis became fully grown in all regions. On rabbit embryos, Sultan et al. (2018) discovered that the muscularis mucosa initially appears as a thin and uneven layer of circular smooth muscle fibers on day 23. On the other hand, the latter author added that the tunica muscularis emerged during the middle of the gestation period and reached full development by day 27 of gestation.

The current study demonstrated that the gastric mucosal folds of rabbit embryos began to appear on day 22 of development and reached full development on day 26. Sultan et al. (2018) and Soliman et al. (2020) also made a comparable observation regarding the development of gastric mucosal folds in rabbit embryos

The current study indicated that as the gestation period advanced, there was a significant increase in the mRNA expression level of *GATA4* in the embryonic stomach. The expression of the *GATA4* gene showed a substantial increase on days 20, 22, and 26 of gestation. These findings interpreted the distinct role of *GATA4* in specifying the development of the glandular stomach into the columnar glandular epithelium (Jacobsen et al., 2002; Thompson et al., 2018).

This study was directed to enhance the understanding of the development of the stomach in rabbit fetuses of different age groups. Specifically, the authors focused on the development of the stomach primordia from the gut tube,

the lining epithelium, gastric pits and glands, and the appearance of gastric mucosal folds. Additionally, this study investigated the molecular characteristics of gastric epithelial cells, including the specific expression of *GATA4* in the region where the stomach was expected to be formed.

Thus, studying the progression in a normal stomach could enhance the comprehension of the underlying causes of gastric cancer, a prevalent contributor to global cancer mortality. An in-depth comprehension of the mechanisms that regulate the determination of cells in the gastric epithelium throughout development may be crucial for interpreting the cause of diseases, particularly the metaplastic alterations that occur following *Helicobacter pylori* infection (Willet and Mills, 2016).

CONCLUSION

A precise understanding of rabbit stomach development is essential for a comprehensive understanding, diagnosis, and treatment of several clinical problems, including atrophic gastritis, as well as conditions related to abnormal stomach formation. Hence, this rabbit study model holds significant potential for enhancing the comprehension of human stomach development, advancing the understanding of infectious diseases, enhancing pharmacology and toxicology research, and facilitating innovative approaches in tissue engineering and personalized medicine for the treatment of gastrointestinal diseases. Future research could explore the expression and function of other transcription factors, signaling molecules, and genes that are known to be involved in stomach development.

DECLARATIONS

Funding

This study is self-funded by the authors.

Authors' contributions

Doaa Nouh designed, supervised the laboratory work, prepared the final version of the manuscript, and submission of the manuscript. Esraa Elsheikh participated in the study design, sample collection and preparation, histological interpretation, and preparation of the manuscript. Maha Kilany contributed to the study design, histological examination, interpretation, and preparation of the manuscript. Nagwa Ibrahim participated in gene expression data acquisition, statistical analysis, and preparation of the manuscript. Eman Elsheikh contributed to the material preparation, data collection, and preparation of the manuscript. All authors read and approved the final version of the manuscript to be published.

Competing interests

The authors declare no conflict of interest.

Ethical considerations

The authors declare that this manuscript is original and is not being considered elsewhere for publication. Other ethical issues, including consent to publish, misconduct, fabrication of data, and redundancy, have been checked by all authors.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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