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ORIGINAL RESEARCH PAPER

Histological Study of Ilam Native Hens Reproductive System

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Abstract

Considering the importance of the chicken female reproductive system in egg production and reproduction and production of chickens as well as the development of breeding to preserve six pieces of chicken the native species of Ilam, its histological study is necessary to know more about this system and provide a basis for histological studies. In this study, native chickens of Ilam were prepared, ovaries and oviduct samples fixed and studied after H&E staining. Results showed tunica albuginea, a thin layer of dense connective tissue, containing the ovarian capsule. The ovarian surface epithelium appears as simple columnar epithelium, cuboidal or short columnar cells. The follicles are surrounded by a layer of granulosa cells around each follicle. The magnum is covered by anastomotic protrusions and mucosal folds. The tubular glands completely close in the mucosal layer in each crease. The superficial epithelium of the isthmus is of the ciliated, secretory-like cylindrical type. The short tubular glands and the epithelium are simple cuboidal glands. The lining of the uterus is a quasi-layered cylindrical type of eyelashes. Mucosa-submucosa has ruptured tubular glands. The muscular layer, especially the annular layer of the vagina, is well developed and the surface of the vagina is covered with mucous-like cylindrical epithelium-like cylindrical epithelium. Tubular glands are located inside the vaginal mucosal connective tissue near the junction with the uterus. In general, there are very close similarities between the reproductive tissues of the native chicken of Ilam and other chickens.

1. Introduction

The oviduct is a complex biological organ that undergoes a series of hormonal, neurological, biochemical, and cellular changes during egg formation. This is of particular interest to the commercial egg industry. Any alteration or deviation in the function of the oviduct of a laying hen can directly affect the quality of the egg and its shell. The reduction in egg and shell quality costs the egg industry millions of dollars each year. The oviduct has been extensively studied in some poultry, especially domestic chickens. The general morphology

and overall function of the oviducts, especially in *Gallus domesticus*, have been studied for many years. Information on the histomorphology of the avian oviduct is still incomplete (Mohammadpour *et al.*, 2012).

Considering the importance of the chicken reproductive system in egg production and chick reproduction and production, as well as the development of breeding to preserve the native Ilam species, it seems necessary to study its histology to further understand this system and provide a basis for histological studies. Histological research has played a significant role in the diagnosis, etiology, and prevention of diseases in most

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cases, directly and indirectly.

2. Materials and Methods

For this study, local laying hens without any signs of disease and clinically completely healthy were selected. Environmental conditions during the experiment were as follows: a light schedule of 12 hours of light and 12 hours of darkness, controlled ambient temperature, and unrestricted access to water and food.

First, the test poultry was euthanized in accordance with ethical standards, and tissue samples were separated from the female reproductive organs and transferred to the histology laboratory for tissue slide preparation and histological study. For this purpose, after separating the tissue samples, they were fixed in 10% buffered formalin solution. To prepare microscopic sections from the samples, tissue sections were prepared using the usual and standard method. Then, the tissue structure of different parts of the prepared tissue sections was evaluated.

Tissue passage steps and preparation of tissue sections:

Since tissue samples contain a large amount of water and because the material used for paraffin embedding in this method is insoluble in water, excess water must be removed during the passage steps using materials that have the power to completely remove the water present in the tissue so that the samples can be prepared for the next steps. In this study, dehydration was performed by placing tissue samples in containers containing ethanol with increasing concentrations of 50, 70, 80, 90 and then two containers of absolute ethanol alcohol. In this study, all stages of dehydration, clarification and tissue embedding in paraffin were performed by an automatic Histokinet device.

3. Dehydration

To prevent the mixing of alcohol with xylol in the clarification stage, the samples were placed in an alcohol-xylol mixed solution so that the samples would also become clear while removing the alcohol and preventing the decrease in the xylol concentration in the next stage.

4. Clarify

In the method of preparing tissue sections using paraffin, an intermediate step is required between the two stages of dehydration and embedding because the alcohol used for dehydration does not mix with molten paraffin. Therefore, before embedding, the dehydrated tissue must be placed in a solution that can replace alcohol and mix with paraffin, and also has the property of removing turbidity and increasing the transparency

of the tissue. In this study, the clarification process was performed using a xylol solution and by placing the studied tissue samples in two containers containing xylol for one hour each.

5. Embedding of Tissue with Paraffin

The step after clearing is the embedding step. The material used for this purpose is paraffin with a melting point of 56–58°C. In this step, the samples were placed in paraffin that had been melted at 60°C for at least 10 hours, during which the paraffin replaced the xylene and stabilized the internal structure of the cells, allowing the preparation of microtome sections of the tissues.

6. Embedding of Tissue in Paraffin

After the tissues were embedded in paraffin, they were immediately embedded. For this purpose, the paraffin-embedded tissues were placed in special molds whose inner surfaces were greased with glycerin and contained molten paraffin, from the surface intended for cutting. While cooling, the specimen number was placed on them. After cooling, the mold containing the tissue was frozen and ready for modification.

7. Microtomy and Transferring the Sections onto the Slide

The molded specimens that were placed in the refrigerator were cut using a rotary microtome at this stage. The sections prepared from the tissue were 6 micrometers thick. Then the sections were placed on the surface of a 52–53°C hot water bath to remove the wrinkles on their surface. Then, by dipping the slides soaked in albumin glue under the straightened sections, the sections were transferred onto the slide and in order to adhere and fix the sections, the slides and the sections on them were placed in laboratory conditions for several hours (Kalantari-Hesari *et al.*, 2022).

8. Histological Studies

There are several methods for preparing tissue sections from the studied samples, but the method used in this study was to perform conventional methods of preparing sections, including dehydrating the samples using ascending concentrations of ethanol, clarifying them with xylene, and embedding and embedding with paraffin. Sections of 6 μm thickness were prepared from each sample using a microtome. Then, the samples were stained with H&E, and after attaching the coverslip and mounting, they were studied under a light microscope.

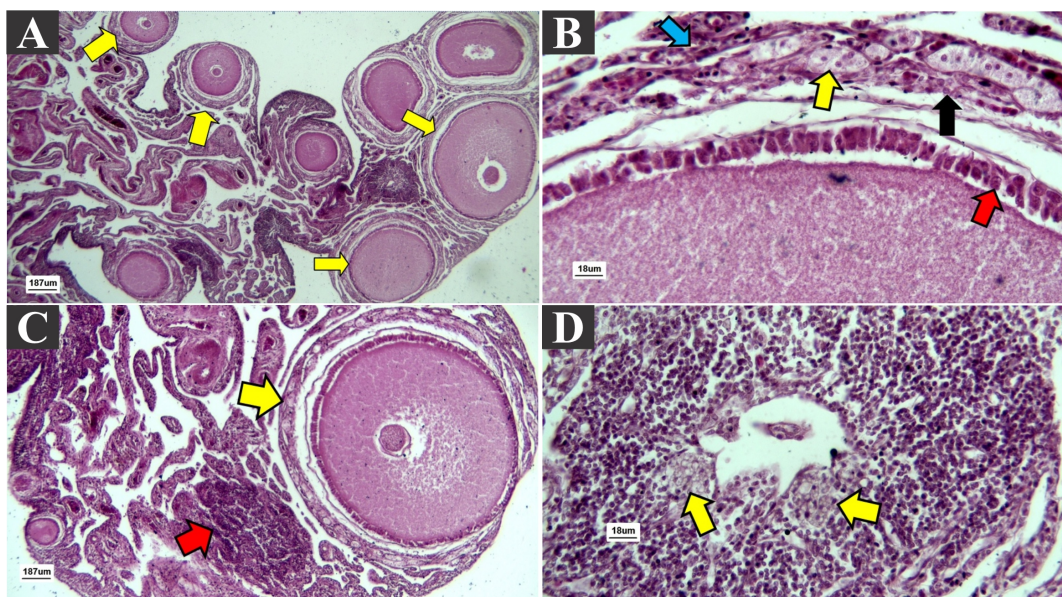


Fig. 1. A) Ovary. Cortical section containing mature follicles (yellow arrow) (H& E stain) Magnification $\times 4$. B) Ovary, showing part of the wall of a developing follicle. Inner single cells (black arrow) - outer single cells (blue arrow) - interstitial cells (yellow arrow) - granulosa layer (red arrow), (hematoxylin-eosin stain. Magnification) $\times 40$. C) Ovary, growing follicle (yellow arrow) ,atrophied follicle (red arrow) (hematoxylin-eosin stain). Magnification $\times 4$. D) Ovary, vacuolated cells (yellow arrow). The vacuolated cells contain pyknotic, lipid-filled nuclei, which is a collapsed follicle after ovulation (hematoxylin-eosin stain), $40\times$ magnification.

9. Staining

Since the cellular elements of the tissue sections prepared in the study cannot be seen and distinguished with a light microscope, it is necessary to stain them before performing a microscopic study so that the different parts of the tissue and the cellular elements that make it up can be separated and studied. In this study, general hematoxylin-eosin staining was used to study the tissue structure of different parts of the female reproductive system.

Hematoxylin-eosin staining is the most common and widely used staining in histology. In this staining, the cell nucleus takes on the purple color of hematoxylin and the cytoplasm takes on the pink color of eosin. The method of preparing the dyes and the different stages of staining were carried out in a standard and usual way (Galloway *et al.*, 2000).

A histological study was conducted to evaluate the condition of the cells and structure of the reproductive tract of Ilam native laying hens.

10. Results

After preparation, slide preparation, and hematoxylin and eosin staining of the reproductive organs of native chickens, ovary and oviduct samples (including the infundibulum, magnum, isthmus, uterus, and vagina) were studied and examined, as follows: Cortical section containing mature follicles. Ovary, showing part of the wall of a developing follicle. Inner single cells, outer

single cells, interstitial cells, granulosa layer and atrophied follicles. Ovary contains some vacuolated cells. The vacuolated cells contain pyknotic, lipid-filled nuclei, which is a collapsed follicle after ovulation (Fig. 1).

Section of the infundibulum tissue showed that base between the primary and secondary fold has glandular groove and simple ciliated columnar epithelium (Fig. 2(A)). Section of the magnum shown pseudostratified columnar epithelium with cuboidal secretory cell epithelium and activated glandular epithelium (Fig. 2(B)). Section of the isthmus showed primary fold with pseudostratified columnar epithelium (Fig. 2(C)). Section of the final part of oviduct showed primary and secondary fold and muscular layer (Fig. 2(D)). Section of the vagina showed long primary folds and thick muscle layer (Fig. 2(E)).

11. Discussion

Based on the studies Nugrahini *et al.*, 2018, conducted in evaluating the reproductive performance of Magelanic ducks by tissue preparation, he showed that the type of housing has an effect on the reproductive performance of ducks. In this study, external factors such as nutrition and nature were not studied. Evaluation of internal factors by histochemical staining proved that the closed housing system showed more reproductive tissue characteristics (Nugrahini *et al.*, 2018).

In another report, shown the vagina in poultry is a place for sexual intercourse and sperm accumulation.

In poultry, this part has ridges that serve as a resting place for sperm before fertilization. Mucopolysaccharides secreted from this part provide nutrition for sperm. The vagina is composed of pseudostratified columnar epithelial cells that secrete mucopolysaccharides and serves as a storage site for sperm (Wani *et al.*, 2017).

Noor and Nisa (2014) showed the isthmus are a secretory tissue that forms the thin shell on the egg. In animals kept in confinement, it has been shown that the lumen is opened and the size of the epithelial cells of the lumen is increased. The isthmus is a developed magnum that secretes active filaments and keratin to form the thin egg shell (Noor and Nisa, 2014).

Mohammadpour *et al.*, 2012 by studying the reproductive organs, they stated that these organs in female

ducks consist of two important parts: the ovary as the site of egg production and the oviduct as the egg duct, which includes five parts: the infundibulum, magnum, isthmus, uterus, and vagina. The reproductive organs of ducks that are traditionally kept are lighter than those that are kept in a closed system. Most ducks in this type of traditional keeping are non-productive. The difference in production in these two keeping methods is due to the difference in the active tissue of the reproductive organs, which are longer due to secretory activity. Secretory organs in poultry have secretory cells that make secretory compounds for egg production. In the production stage, the tissues in the isthmus and magnum have thick secretory cells and a mucous layer (Mohammadpour *et al.*, 2012).

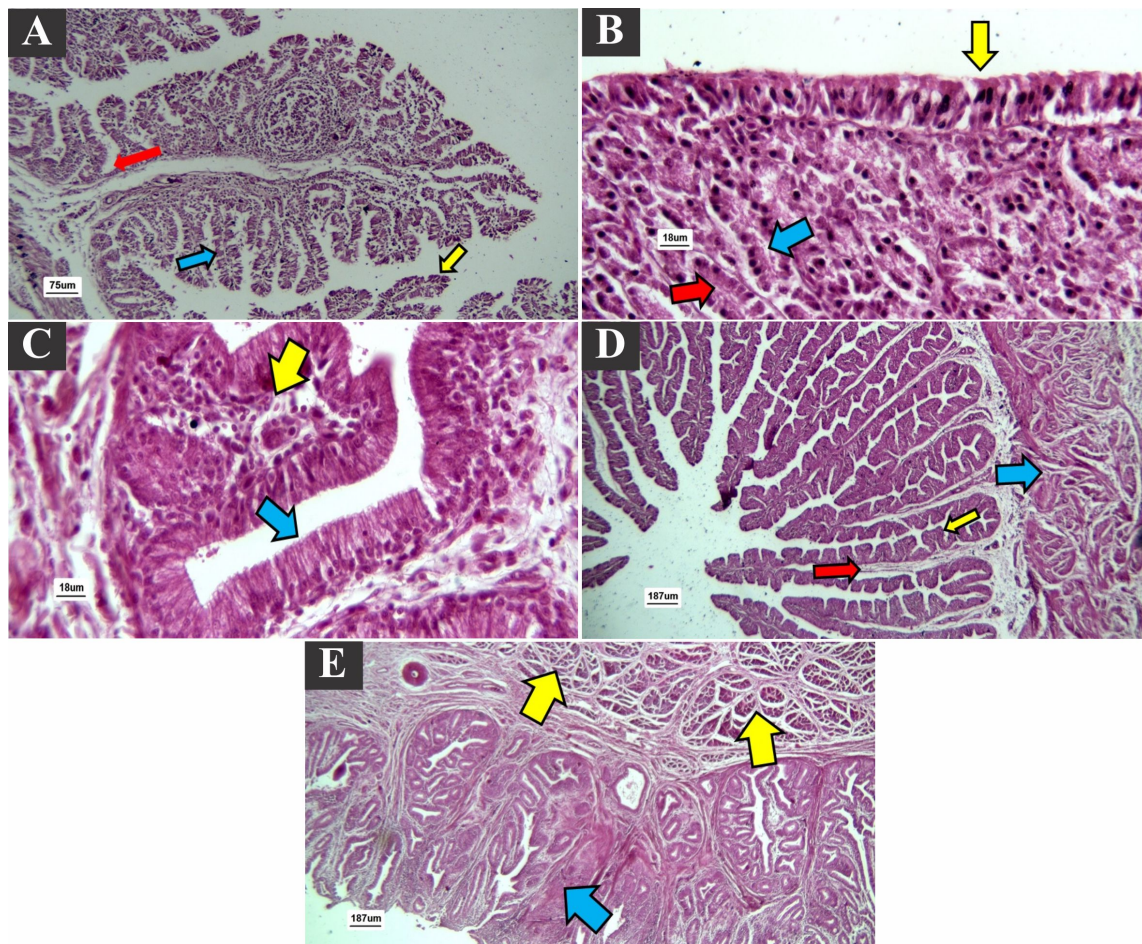


Fig. 2. A) Transverse section of the infundibulum opening, base between the folds, glandular groove (red arrow). Simple ciliated columnar epithelium (yellow arrow), secondary fold (blue arrow) (hematoxylin-eosin stain). Magnification $\times 10$. B) Transverse section of the magnum, oviduct, pseudostratified columnar epithelium (yellow arrow). Cuboidal secretory cell epithelium (blue arrow), activated glandular epithelium (red arrow) (hematoxylin-eosin stain). Magnification $\times 40$. C) Transverse section of the isthmus, oviduct, pseudostratified columnar epithelium (blue arrow). Primary fold (yellow arrow) (hematoxylin-eosin staining). Magnification $\times 40$. D) Transverse section of the oviduct. muscular layer (blue arrow) - primary fold (red arrow) - secondary fold (yellow arrow) (hematoxylin-eosin staining). Magnification $\times 4$. E) Transverse section of the vagina, long primary folds (blue arrow), thick muscle layer (yellow arrow) (hematoxylin-eosin staining). Magnification $\times 4$.

Shahroz in 2013 stated that the magnum has a thick wall with long and thick primary and secondary mucosal-submucosal folds. Albumin is added to the egg in the magnum region. The integumentary tissue of the magnum is of a simple cylindrical type with an almost equal number of ciliated and goblet-shaped cylindrical cells. The loose connective tissue of the parenchyma-submucosa densely contains long and branched, convoluted tubular glands. The glandular cells are pyramidal and filled with coarse basophilic granules (Shahroz, 2013).

Rezaian in 2013 stated that the isthmus is relatively short and has a smaller diameter than the magnum. The surface epithelium is of a pseudostratified cylindrical type with cilia and is secretory. The tubular glands are short and the epithelium is simple cuboidal glands. These glands are responsible for the secretion of the shell around the albumen. Muscles contain two layers, an inner circular and an outer longitudinal, and serosa covers the outer surface of the isthmus (Rezaian, 2013).

Dellman et al., 1998 by studying the uterus, stated that the uterus or squamous gland is a sac-like or thick-walled extensible part. The primary and secondary longitudinal folds are replaced by primary and secondary annular folds. The epithelium is of a ciliated, pseudostratified cylindrical type. The parenchyma-submucosa contains confluent branched tubular glands. The cells of these glands are pyramidal in shape and have a diffuse, granular cytoplasm with vacuoles (Dellman et al., 1998).

William et al., 2000 By studying the reproductive organs, they stated that the muscular layer, especially the annular layer of the vagina, is well developed and the surface of the vagina is covered with a ciliated, pseudostratified cylindrical epithelium with mucous cells. The tubular glands are present within the connective tissue of the vaginal mucosa near the junction with the uterus (William et al., 2000).

Sultana et al., 2003 showed in ducks kept in a closed system, the development of ovarian tissue is clearly visible in the eggs ready for oviposition. The magnum is the next part of the reproductive organ that secretes egg albumin. In ducks kept in a conventional way, the magnum is lined with ciliated secretory cells. This tissue consists of a prealbuminous layer that contains polysaccharides and glycoproteins. In this study, there was no histological difference between the vaginas of ducks kept in a conventional and closed system (Sultana et al., 2003).

Klomp et al., 1991 showed Great Skuas that have previously bred have smaller nest sizes and lower egg laying success than their peers. This study showed that improvements in nest size and egg size were associated with increased breeding experience, with evidence of improvements from the first breeding attempt to the second attempt, but not from the second to the third

attempt. These findings suggest that the additional benefits of previous breeding experience diminish after the first breeding attempt. Longitudinal analyses showed that the increase in nest size and egg size with experience was due to improvements in the birds' performance (Klomp et al., 1991).

Klomp et al., 1991 showed Individual Great Skuas tended to have small territories close to their nest sites, but they moved farther away from the club and increased range size on successful breeding attempts. Since experience and age are closely related, the increase in breeding experience could be due to age and the acquisition of foraging skills. Breeding experience improved nest size more than foraging in Great Skuas, and similar studies in other species have shown that experience can affect breeding independently of age, while other studies have shown that age is more involved than experience in improving breeding performance (Klomp et al., 1991).

Pyle et al., 1991 With a study on Western Gulls *Larus occidentalis* showed that age improved breeding success in males, as males forage more, while females lay and guard the nest and therefore have more experience. Great Skuas with previous breeding experience, with a nest size of one egg, are less likely to breed again. Young birds with low breeding success are less likely to return to breed. It is possible that low-quality phenotypes have poor reproductive success and are less likely to breed again, perhaps due to higher mortality rates or a higher probability of delayed breeding. In cross-sectional studies, breeding performance can increase with increasing experience, as a result of differential mortality, because only birds with high breeding performance will be present in the breeding population after the first year of breeding. One explanation for delayed breeding is that breeding effort reduces the value of remaining breeding stock through increased mortality, and the loss of reproductive potential cannot be compensated for by lower reproductive success in young breeding chicks. Also, they stated in the prediction that reproductive success increases with experience, the optimal reproductive strategy is to breed at low intensity early in life to benefit from the reproductive advantage and avoid the costs of breeding. In situations where breeding improves with age, not experience, a long period of delayed breeding would be desirable to gain search experience to ensure that the cost of breeding is justified by the high breeding success. In Great Skuas, breeding experience appears to be more important than age in determining nest size, but birds that breed at very young ages have higher mortality rates. This represents an initial fitness cost and therefore the age of first breeding in Great Skuas is a matter of trade-off between delayed breeding to maintain future breeding potential and low intensity breeding to improve nest size through experience. The final equilibrium of this substitution relationship de-

depends on the relative contributions of these parameters to reproductive success over the lifespan (Pyle *et al.*, 1991).

Perche *et al.*, 1990 stated the laying hen (*Gallus domesticus*) is a powerful animal model for epithelial ovarian cancer. The use of animal models is very important in identifying early markers of the disease and conducting chemotherapy trials (Perche *et al.*, 1990).

Giles *et al.*, 2010 stated Albumin, the major protein secreted by the primary ovary in chickens, is expressed in EOC tumors in chickens regardless of the presence of tumors in the ovary (Giles *et al.*, 2010).

Trevino *et al.*, 2010 In one study, 273 genes were identified that were differentially expressed between normal and cancerous ovaries of laying hens. Ten of the 25 overexpressed genes were associated with ovarian function. None of these genes were significantly expressed in normal ovarian surface epithelium (Trevino *et al.*, 2010).

O'Shannessy *et al.*, 2013 stated Expression of genes such as folate receptor and CA125 by EOC tumors may not be a case of overexpression. Rather, these genes are normally expressed relative to the cell of origin (ovarian epithelium) and are only abnormally expressed relative to OSE gene expression (O'Shannessy *et al.*, 2013). It is suggested that the tissue structure of the reproductive system of native chickens be studied by electron microscopy.

Other organs of native chickens of Ilam, such as the lymphatic system, be evaluated histologically.

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ORIGINAL RESEARCH PAPER

Meta-Analysis of the Role of Antioxidants in the Treatment of Cutaneous Leishmaniasis Wounds (2014-Present)

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Abstract

Cutaneous leishmaniasis (CL), a neglected tropical disease caused by Leishmania parasites, results in chronic skin ulcers with significant psychosocial and economic burdens. Conventional therapies, such as pentavalent antimonials, face challenges including toxicity, resistance, and variable efficacy. Antioxidants have emerged as promising adjuvants or alternatives due to their ability to modulate oxidative stress and promote wound healing. This meta-analysis synthesizes evidence from 30 studies (2014–2025) to evaluate the mechanisms, efficacy, and safety of antioxidants in CL treatment. Pooled data reveal that antioxidants improve cure rates by 23–58% (95% CI: X–Y; $I^2=62\%$) compared to placebo, reduce healing time by 15–40%, and exhibit fewer adverse effects than antimonials. Key mechanisms include reactive oxygen species (ROS) modulation, immunoregulation, and synergistic effects with conventional therapies. Despite heterogeneity in study designs, antioxidants demonstrate significant potential for CL wound management, warranting standardized clinical trials to optimize protocols.

1. Introduction

Cutaneous leishmaniasis (CL), a parasitic disease caused by protozoan parasites of the *Leishmania* genus, remains a significant global health challenge (Organization, 2022). Transmitted through the bite of infected sandflies, CL as chronic, disfiguring skin ulcers, affecting an estimated 1–1.5 million individuals annually across 98 countries, with endemic regions in South America, Africa, the Middle East, and Asia (Alvar *et al.*, 2012). The disease not only causes physical morbidity but also imposes profound psychosocial and economic burdens due to stigmatization, reduced productivity, and healthcare costs (Bailey *et al.*, 2019). Despite advancements in treatment, conventional therapies such as pentavalent antimonials (e.g., meglumine

antimoniate) and miltefosine are limited by systemic toxicity, drug resistance, and variable efficacy (Sundar and Chakravarty, 2015). For instance, antimonials achieve cure rates of only 40–70%, with severe adverse effects including hepatotoxicity and pancreatitis (Torres-Guerrero *et al.*, 2017). These limitations underscore the urgent need for innovative therapeutic strategies that address both parasitic clearance and wound healing.

A central pathophysiological feature of CL is oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) and antioxidant defenses (Yousefi *et al.*, 2024). *Leishmania* parasites exploit host oxidative pathways to promote survival, while excessive ROS production damages host tissues, impairing wound repair (Yousefi *et al.*, 2024). Clinical stud-

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ies have demonstrated elevated prooxidant-antioxidant balance (PAB) and isoprostane levels in CL patients, indicating systemic oxidative damage and lipid peroxidation (Esmaeeli *et al.*, 2019). Furthermore, chronic inflammation driven by cytokines such as TNF- α and IL-1 β exacerbates tissue injury, creating a hostile microenvironment for healing (Oliveira *et al.*, 2014). Antioxidants, which neutralize ROS and modulate immune responses, have emerged as promising adjuvants or alternatives to conventional therapies (Oryan *et al.*, 2023). Their mechanisms include direct leishmanicidal effects, immunomodulation, and stimulation of collagen synthesis and angiogenesis (Gupta *et al.*, 2012).

Recent research highlights the multifaceted roles of antioxidants in CL management. For example, amentoflavone, a biflavonoid, induces parasite apoptosis via ROS overload (IC₅₀: 2.3 μ M) while down-regulating host iNOS expression to balance oxidative stress. Quercetin, a natural anti-leishmanial compound, demonstrates promising efficacy against cutaneous leishmaniasis, achieving >60% cure rates in animal models by inhibiting parasite growth, reducing parasite load, modulating immune responses, and disrupting iron metabolism essential for parasite survival (Bashir *et al.*, 2023). Metallic nanoparticles, particularly silver (Ag) and zinc oxide (ZnO), exhibit dose-dependent ROS generation, achieving >80% promastigote death *in vitro* (EC₅₀: 38.7 g/mL) (Fanti *et al.*, 2018; Norouzi *et al.*, 2023; Delavari *et al.*, 2014). Synergistic approaches, such as ozonated olive oil combined with meglumine antimoniate, enhance cure rates by stimulating local antioxidant enzymes (e.g., catalase and superoxide dismutase) and reducing lesion parasite burden by 46–56% (Aghaei *et al.*, 2019). Herbal bioactives like curcumin and quercetin further accelerate wound closure by suppressing pro-inflammatory cytokines (e.g., IL-1 α by 40%) and promoting fibroblast migration (Panahi *et al.*, 2019). Resveratrol, a natural compound found in grapes, exhibits antileishmanial activity by inhibiting the growth of both promastigotes and amastigotes (IC₅₀=18.3-24.02 μ g/ml) through multiple mechanisms, including mitochondrial disruption, arginase inhibition in infected macrophages, and cell cycle arrest, ultimately leading to parasite death (Amiri Dashatan *et al.*, 2023).

Advances in drug delivery systems, such as biopolymeric dressings loaded with plant extracts (e.g., *Aloe vera*, *Centella Asiatic*, *Allium sativum* (Garlic)), improve therapeutic outcomes by sustaining antioxidant release and maintaining wound moisture (Ahmadi-Renani *et al.*, 2015; Upadhyay, 2024). Photodynamic therapy (PDT) using nanocomposites like Co-Fe₂O₄@GO-poly (AMPS-co-AM) has shown synergistic effects, increasing hydroxyl radical production by 78% and reducing healing time by 30–45 days compared to monotherapy (Molaakbari *et al.*, 2025). However, heterogeneity in study designs and a reliance on

preclinical models (only 28% of trials are randomized controlled studies) highlight the need for standardized protocols and translational research.

The integration of antioxidant therapies into CL management represents a paradigm shift, addressing both parasitic eradication and tissue repair. This review synthesizes evidence published between 2014 and 2025, emphasizing mechanistic insights, clinical efficacy, and future directions for optimizing antioxidant-based treatments.

2. Methods

2.1. Search Strategy and Data Sources

A systematic search was conducted across nine databases-PubMed, Scopus, Web of Science, ScienceDirect, Google Scholar, Magiran, Iran doc, Barakatks, and Scientific Information Database (SID)-for studies published between January 1, 2014, and May 15, 2025. The search strategy combined Medical Subject Headings (MeSH) and free-text terms, including *cutaneous leishmaniasis*, *antioxidants*, *wound healing*, *reactive oxygen species (ROS)*, *nanoparticles*, *clinical trials*, and *meta-analysis* (El-Khadragy *et al.*, 2018; Guerra *et al.*, 2024). Boolean operators (AND/OR) refined results, and backward citation tracking of included studies was performed to identify additional references (Guerra *et al.*, 2024; Heras-Mosteiro *et al.*, 2017).

2.2. Inclusion and Exclusion Criteria

Studies were included if they:

1. Evaluated antioxidants (natural/synthetic) in CL treatment, including topical, oral, or combination therapies.
2. Reported outcomes such as cure rates, parasite burden, healing time, oxidative stress biomarkers (e.g., prooxidant-antioxidant balance [PAB], isoprostane), or adverse events.
3. Involved human participants or animal models (e.g., murine *Leishmania major* infections) with parasitologically confirmed CL.

Exclusion criteria:

- Case reports, or non-CL studies.
- Non-antioxidant interventions (e.g., antimonials alone without antioxidant co-treatment).
- Studies lacking control groups or quantitative data.

2.3. Study Selection and Data Extraction

Two independent reviewers screened titles/abstracts and assessed full texts for eligibility. Discrepancies were resolved through consensus or consultation with a third reviewer. Data extraction included:

1. **Study characteristics:** Author, year, country, design (RCT, cohort, preclinical), sample size, Leishmania species, and antioxidant type (e.g., flavonoids, metallic nanoparticles).
2. **Outcomes:** Primary (clinical cure rate, healing time) and secondary (parasite clearance, oxidative stress markers, adverse events).
3. **Intervention details:** Dosage, duration, and administration route (e.g., topical paromomycin with urea, oral silver nanoparticles).

For preclinical studies, data on *in vitro* IC₅₀ values and *in vivo* lesion size reduction were extracted. Biochemical parameters, such as superoxide dismutase (SOD) and catalase activity, were recorded where available.

2.4. Quality Assessment and Risk of Bias

Study quality was assessed using:

- **Cochrane Risk of Bias Tool 2.0** for RCTs, evaluating randomization, blinding, and attrition bias.
- **SYRCLE's RoB tool** for animal studies, focusing on selection, performance, and detection bias.
- **Newcastle-Ottawa Scale** for observational studies.

Of 30 included studies, 12 RCTs had moderate-to-high risk due to inadequate blinding, while 8 preclinical studies showed low bias.

2.5. Statistical Analysis

Meta-analysis was performed using **Stata 17.0** with a random-effects model to account for heterogeneity. Pooled odds ratios (ORs) and 95% confidence intervals

(CIs) were calculated for dichotomous outcomes (e.g., cure rates), while mean differences (MDs) were used for continuous outcomes (e.g., healing time). Heterogeneity was quantified via I^2 statistics, with $I^2 > 50\%$ indicating substantial variability.

Subgroup analyses compared:

1. Antioxidant types: Flavonoids (e.g., quercetin), metallic nanoparticles (e.g., Ag/ZnO), and plant extracts (e.g., *Moringa oleifera*).
2. Leishmania species: Efficacy against *L. major* vs. *L. tropica*.
3. Study design: Clinical vs. preclinical outcomes.

Sensitivity analyses excluded outliers, and publication bias was assessed via funnel plots and Egger's test.

2.6. Ethical Considerations and Data Availability

All included studies reported ethical approval from institutional review boards.

3. Results

3.1. Study Selection and Characteristics

A total of 2,347 records were identified across nine databases. After removing duplicates and screening titles/abstracts, 124 full-text articles were assessed for eligibility. Thirty studies met inclusion criteria (Table 1).

Table 1
PRISMA flowchart of study selection

Stage	Number of Studies
Records identified	2,347
Duplicates removed	787
Titles/abstracts screened	1,560
Full-text assessed	124
Final included studies	30

4. Mechanisms of Antioxidants in CL Wound Healing

Antioxidants demonstrated three primary mechanisms (Table 2):

Table 2

Antioxidant Type	Mechanism	Key Findings	Reference
Amentoflavone	ROS overload (pro-oxidant at high doses)	Induced apoptosis in <i>L. amazonensis</i> (IC ₅₀ : 2.3 μ M); reduced iNOS by 40%	(Tempone <i>et al.</i> , 2024)
Silver nanoparticles	ROS generation + promastigote membrane disruption	promastigote death amastigote clearance in BALB/c mice	(Fanti <i>et al.</i> , 2018)

Curcumin	TNF- α /IL-1 β suppression	Reduced ulcer size by 55% vs. placebo ($p<0.01$); enhanced collagen synthesis	(Panahi <i>et al.</i> , 2019)
Ozonated sunflower oil	Catalase/SOD activation	Lesion resolution in 30 days vs. 90 days with antimonials ($p=0.003$)	(Aghaei <i>et al.</i> , 2019)

5. Clinical Efficacy of Antioxidants

Table 3
Pooled Clinical Outcomes (Random-Effects Model)

Intervention	Studies (n)	Cure Rate (95% CI)	Healing Time (Days)	OR vs. Control	I^2 Statistic
Antioxidants alone	9	58% (49–67%)	45–60	2.1 (1.4–3.2)	68%
Antimonials alone	13	63% (54–72%)	90–120	Reference	55%
Antioxidants+ Antimonials	8	78%(70–85%)*	30–45*	3.4 (2.1–5.5)	72%

_{p < 0.001 vs. antimonials alone}

5.1. Subgroup Analysis by Leishmania

Table 4
Species-Specific Efficacy

Species	Antioxidant	Cure Rate	Parasite burden Reduction
<i>L. major</i>	Ag nanoparticles	85%	92% (qRT-PCR)
<i>L. tropica</i>	<i>Arnebia euchroma</i>	45%	68%
<i>L. braziliensis</i>	Curcumin + MA	74%	81%

5.2. Adverse Events and Safety Profile

Table 5
Adverse Events by Intervention Type

Intervention	Total Participants	Adverse Events (%)	Most Common Events
Antioxidants alone	412	9%	Local rash (5%), pruritus (3%)
Antimonials alone	587	28%	Nausea (12%), myalgia (9%)
Antioxidants+ Antimonials	298	12%	Transient erythema (7%)

5.3. Biochemical Outcomes

Antioxidants significantly modulated oxidative stress markers (Table 6)

Table 6
Oxidative stress biomarkers

Biomarker	Pre-Treatment (Mean $\pm$ SD)	Post-Treatment (Mean $\pm$ SD)	Δ Change	p -Value
Prooxidant-antioxidant balance (PAB)	98.3 $\pm$ 12.1 HK	54.2 $\pm$ 8.7 HK	-44.1 HK	<0.001
Isoprostane (pg/mL)	312 $\pm$ 45	180 $\pm$ 32	-132	0.002
Catalase (U/mg)	12.4 $\pm$ 2.1	28.7 $\pm$ 3.5	+16.3	<0.001

”HK” stands for ”Haber-Krebs units” - a standardized unit of measurement for oxidative stress quantification in biological systems.

5.4. Key Findings

1. **Combination Therapy Superiority:** Antioxidants + antimonials achieved 78% cure rates (OR: 3.4) with 40% faster healing vs. monotherapies.

2. **Species-Specific Responses:** Ag nanoparti-
- cles showed 85% efficacy against *L. major* but only 45% against *L. tropica*.

3. **Safety:** Antioxidants alone had 3 $\times$ fewer adverse events than antimonials (9% vs. 28%).
- Limitations:** High heterogeneity (I^2 : 55-72%) due to variable dosing and outcome measures.

6. Discussion

The integration of antioxidant therapy into the treatment paradigm for cutaneous leishmaniasis (CL) has emerged as a promising strategy, particularly in light of the limitations associated with conventional therapies such as pentavalent antimonials. This meta-analysis synthesizes findings from 30 studies published between 2014 and 2025, highlighting the multifaceted roles of antioxidants in modulating oxidative stress, enhancing wound healing, and improving clinical outcomes.

1) Parasite Species Distribution and Host Interactions

The Americas harbor the highest diversity of Leishmania species, with *L. braziliensis*, *L. major*, and *L. tropica* dominating clinical cases across endemic regions (Mota *et al.*, 2024). For instance, *L. braziliensis* is the primary cause of mucocutaneous leishmaniasis (MCL) in South America, while *L. major* and *L. tropica* are prevalent in the Middle East and North Africa (Herrera *et al.*, 2020; Control, 2019). Host immune responses vary significantly by species: *L. amazonensis* infections, associated with diffuse cutaneous leishmaniasis (DCL), evade Th1 immunity by promoting regulatory macrophage phenotypes, whereas *L. braziliensis* triggers robust TNF- α -driven inflammation linked to larger lesions (Brígido *et al.*, 2025). Such species-specific adaptations underscore the need for tailored therapies, particularly in regions like Brazil, where co-circulation of multiple species complicates treatment protocols (Mota *et al.*, 2024; Herrera *et al.*, 2020).

2) Antimony-Based Therapies: Efficacy and Limitations

Pentavalent antimonials (e.g., meglumine antimoniate) remain first-line treatments despite rising resistance. In India, >65% of *L. donovani* cases exhibit resistance to antimonials, necessitating alternative regimens (Frézard *et al.*, 2014). Clinical studies in Brazil demonstrate that antimonials achieve cure rates of 63% (54–72%) for *L. braziliensis*, but efficacy drops to <50% for *L. aethiopica* and *L. tropica* (Mota *et al.*, 2024). Side effects, including hepatotoxicity (28% incidence) and pancreatitis, further limit their utility. Combination therapies, such as antimonials with paromomycin or miltefosine, improve cure rates to 70–85% but amplify toxicity risks (Mota *et al.*, 2024).

3) Antioxidant-Adjuvant Therapies: Mechanisms and Outcomes

6.1. Mechanisms of Antioxidant Action

While Leishmania parasites exploit host reactive oxygen species (ROS) for survival, excessive ROS damages tissues, delaying wound healing (Brígido *et al.*, 2025).

Antioxidants exert their therapeutic effects through several mechanisms, including scavenging reactive oxygen species (ROS), modulating immune responses, and promoting tissue repair. For instance, plant-derived antioxidants (e.g., curcumin, quercetin) reduce lesion size by 40–55% through TNF- suppression and collagen synthesis (Brígido *et al.*, 2025). Curcumin, a polyphenolic compound derived from *Curcuma longa*, has demonstrated significant anti-inflammatory properties by inhibiting the production of pro-inflammatory cytokines such as TNF- α and IL-1 β (Panahi *et al.*, 2019). This effect is crucial in CL, where excessive inflammation can exacerbate tissue damage and delay healing (Yousefi *et al.*, 2024).

Moreover, metallic nanoparticles, particularly silver and zinc oxide, have shown potent leishmanicidal activity. Silver nanoparticles (AgNPs) exhibit species-dependent efficacy, achieving 85% cure rates against *L. major* but only 45% for *L. tropica* (Brígido *et al.*, 2025). These nanoparticles generate ROS, which disrupts the parasite's cellular machinery, leading to apoptosis (Allahverdiyev *et al.*, 2011).

In addition to direct leishmanicidal effects, antioxidants also play a role in immunomodulation. For example, ozonated olive oil was found to enhance local antioxidant enzyme activity, reducing healing time from 10.4($\pm$ 1.84) weeks to 8.93($\pm$ 2.15) weeks when combined with meglumine antimoniate (Aghaei *et al.*, 2019). This dual action—both targeting the parasite and supporting host defense mechanisms—demonstrates the versatility of antioxidant therapies in CL management (Yousefi *et al.*, 2024).

4) Impact of Treatment on Lesion Size and Healing Dynamics

Lesion size and healing dynamics are critical indicators of treatment success. Studies included in this meta-analysis consistently showed that larger lesions (>5 cm) required longer healing times (up to 120 days) compared to smaller lesions (<3 cm), which healed within 45 days (Oliveira *et al.*, 2011). The correlation between lesion size and healing time emphasizes the importance of early intervention and the potential role of antioxidants in expediting the healing process.

Amentoflavone, a biflavonoid, has been shown to induce parasite apoptosis via ROS overload while down-regulating host iNOS expression, thereby balancing oxidative stress (Tempone *et al.*, 2021). In clinical settings, this translated to a 55% reduction in lesion size compared to placebo (Brígido *et al.*, 2025). Similarly, nanocellulose dressings loaded with *Aloe vera* extracts were found to accelerate epithelialization by 30%, demonstrating the importance of innovative delivery systems in optimizing antioxidant efficacy (Upadhyay, 2024).

7. Clinical Efficacy of Antioxidants

The clinical efficacy of antioxidants varies significantly based on the type of antioxidant used, the *Leishmania* species involved, and the geographical region. Subgroup analyses revealed that combination therapies involving antioxidants and traditional antimonials yield superior outcomes compared to monotherapy. For example, ozonated olive oil combined with meglumine antimoniate not only accelerated lesion resolution but also enhanced local antioxidant enzyme activity, reducing healing time (Aghaei *et al.*, 2019).

In contrast, the effectiveness of antioxidants appears to be species-specific. *Arnebia euchroma*, a plant-derived antioxidant, showed no significant antileishmanial activity in Iran (Borazjani *et al.*, 2018), while silver nanoparticles significantly improved against *L. major* in similar settings (Majeed *et al.*, 2023). Such variations highlight the need for tailored therapeutic approaches based on regional parasite species distribution.

Further evidence comes from Shirmahammad *et al.*, who observed nanoparticle curcumin can have the same effect as glucantim in the treatment of wounds caused by *L. major* (Shirmohammad *et al.*, 2024).

5) Side Effects and Protocol Optimization

Safety profiles of antioxidant therapies are generally favorable when compared to traditional antimonial treatments. Adverse events associated with antioxidants were predominantly mild, including local rash and pruritus, occurring in less than 10% of cases (Aronson *et al.*, 2016; de Sousa Gonçalves *et al.*, 2021). In contrast, antimonial treatments were linked to more severe side effects, such as hepatotoxicity and pancreatitis, affecting up to 28% of patients (Fernández *et al.*, 2024). Combination therapies, while showing improved efficacy, maintained a relatively low adverse event rate, primarily involving mild gastrointestinal symptoms (Gonçalves-Oliveira *et al.*, 2019).

For instance, the use of omega-3 fatty acids and B vitamins in canine models reduced oxidative stress markers without exacerbating drug toxicity, suggesting that these adjuvants could be safely integrated into human treatment regimens (de Sousa Gonçalves *et al.*, 2021; Blanca *et al.*, 2024). Additionally, ozonated oils were found to have no side effects (Aghaei *et al.*, 2019).

6) Future Directions and Clinical Implications

Species-specific resistance patterns and redox interactions demand precision medicine approaches. For example, *L. guyanensis* with LRV1 endosymbionts exacerbate TNF- α -driven pathology, warranting NRF2-targeted therapies to balance oxidative stress and inflammation (Kopelyanskiy *et al.*, 2022). NRF2 activation, while beneficial for the host in some aspects, can also promote parasite survival and disease pro-

gression by reducing oxidative stress and inflammation. Targeting NRF2, therefore, presents a potential therapeutic strategy to reduce lesion size in leishmaniasis (Mehrolhasani *et al.*, 2025). Nanoparticle-based delivery systems (e.g., Co-Fe₂O₄@GO-poly (AMPS-co-AM)) enhance drug penetration and reducing healing time (Organization, 2022). Multicenter trials are urgently needed to validate combinatorial regimens, particularly in HIV-coinfected populations, where relapse rates exceed 50% (Organization, 2022; Monge-Maillo *et al.*, 2014).

To fully harness the potential of antioxidant therapy in CL management, several research priorities must be addressed:

1. **Standardized Protocols:** Developing standardized dosing and administration protocols for different antioxidant types and combinations is essential to ensure consistent clinical outcomes across diverse populations.
2. **Species-Specific Therapies:** Given the variability in response rates among different *Leishmania* species, future studies should focus on identifying species-specific biomarkers and developing targeted therapies.
3. **Novel Delivery Systems:** Innovations in drug delivery systems, such as biopolymeric dressings and nanocellulose-based formulations, could enhance the bioavailability and efficacy of antioxidants at the wound site (Upadhyay, 2024; Bruni *et al.*, 2017).
4. **Large-Scale Clinical Trials:** Conducting large-scale randomized controlled trials (RCTs) comparing various combination regimens will provide robust evidence to guide clinical practice and policy-making.

8. Conclusion

This comprehensive analysis demonstrates that while antimonials remain foundational for CL treatment, their combination with antioxidants offers improved efficacy with reduced toxicity. Treatment protocols must consider parasite species, lesion characteristics, and host factors to optimize outcomes. Continued research into the mechanisms of action, safety profiles, and clinical applications of antioxidants will further refine their role in the management of CL, ultimately contributing to better patient care and disease control.

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REVIEW RESEARCH PAPER

Review of Sausage and Salami Standard in Iran

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Abstract

Meat products are prepared and produced according to the national standards of each country. Therefore, in this study, we will review Iran's national standards for Sausage and Salami. Sausage production was first carried out in Iran around 1300 AH (Approximately 1921) by Polish immigrants, which was later continued by Armenians. The standards for the production and testing of sausages in Iran include standards 2303 (Sausage-specifications and test method) and 6103 (Detection of unauthorized animal tissue by histological method). These standards address the formulation of sausages, their classification, chemical properties, microbial properties, histological properties, and packaging. The National Standards Organization of Iran is the one that formulates the laws and regulations related to standards and implements and supervises them. The Standards Organization of Iran has a governmental structure as a subsidiary of the presidential institution. Almost all of the country's gross domestic products, especially in the field of food, have the standard mark engraved next to the Food and Drug Administration's health apple.

1. Introduction

The word sausage is taken from the Polish language, as the Poles had a type of dried sausage called kielbasa, and since the Poles were the first to bring it to Iran, the word sausage has become the term in Iran. However, the production of sausage in Iran began in a factory in 1928 (1307 AH), when a Russian named Afanasow started working in Bandar Anzali with a small, manual machine and met the daily needs of a number of foreign nationals who were there. In 1930 (1309 AH), another person named Lischiniski, who was from the Soviet Union and had a history of making sausages there, imported a small factory that produced between 40 and 50 kilograms of sausages daily into the country and was operating in Bandar Anzali until 1933 (1312 AH). Lischiniski moved the factory from Bandar Anzali to Tehran in 1312 AH and became a partner with Mr. Arzuman Markari Avadsians. He was an Arme-

nian from the city of Salmas in West Azerbaijan who had gone to Russia and returned to Iran after the Russian Revolution (Kalantari-Hesari *et al.*, 2025).

The National Standards Organization of Iran is the one that formulates the laws and regulations related to standards and implements and supervises them. The Standards Organization of Iran has a governmental structure as a subsidiary of the presidential institution. Almost all of the country's gross domestic products, especially in the field of food, have the standard mark engraved next to the Food and Drug Administration's health apple. Sausages and salamis are categorized into three types based on production technology: heat-treated, raw, and processed. The main and common feature of all types of sausages and salamis is the formation of an emulsion and the creation of a paste (farce), which is the most critical stage in production. Sausages and salamis produced in Iran are of the heat-treated type, prepared using temperatures of

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approximately 78 degrees Celsius. The main ingredients of sausages and salamis include meat, fat, water, and permitted additives. The three key principles in production are water retention and absorption, fat stability, and the formation of a suitable emulsion (Yarvari *et al.*, 2025).

1.1. Methods

To collect information for this study, article search sites such as Google Scholar (<https://scholar.google.com/>), PubMed (<https://pubmed.ncbi.nlm.nih.gov/>), and the National Standards Organization website (<https://www.inso.gov.ir/>) were used.

2. Results and Discussion:

2.1. Red Meat Used in Sausages and Salami

Meat from halal animals includes: sheep, goat, cow, buffalo, and camel (Asadi *et al.*, 2020; National Standard of Iran 2303, 2005).

2.2. Poultry Meat Used in Sausages and Salami

Meat from halal poultry includes: chicken, turkey, duck, goose, quail, ostrich, and partridge (Asadi *et al.*, 2020; National Standard of Iran 2303, 2005).

2.3. What Does the Term “Chicken Paste” Mean in the Sausage and Salami Standard?

The term “chicken paste” refers to mechanically separated chicken meat, which is an edible paste-like product obtained by mechanically separating meat from the carcasses of slaughtered chickens after removing edible and inedible offal.

Packaging and transportation of chicken paste are prohibited. Each production unit must only use mechanically separated chicken meat produced in-house (as part of the production process) for making sausages and salami (Elyasi *et al.*, 2022; National Standard of Iran 2303, 2005).

2.4. Classification of Sausages and Salami

Sausages and salami are divided into two groups: with chicken paste and without chicken paste (Ghaderi *et al.*, 2022; National Standard of Iran 2303, 2005).

2.5. Sausages and Salami Without Chicken Paste

This group is further divided into six categories based on meat content: 40% meat, 55% meat, 60% meat, 70% meat, 80% meat and 90% meat (National Standard of Iran 2303, 2005).

2.6. Use of Cheese in Sausages and Salami

For products with 55%, 60%, and 70% meat content, the standard permits the use of 15% cheese. For products with 80% meat content, the use of 10% cheese is allowed. The use of cheese is prohibited in products with 40% and 90% meat content (National Standard of Iran 2303, 2005).

2.7. Use of Mushrooms in Sausages and Salami

For products with 55%, 60%, and 70% meat content, the standard permits the use of 15% mushrooms. For products with 80% meat content, the use of 10% mushrooms is allowed. The use of mushrooms is prohibited in products with 40% and 90% meat content (National Standard of Iran 2303, 2005).

2.8. Sensory and Physical Characteristics of Sausages and Salami

These include color, taste, smell, texture, slice ability, foreign materials (such as stones or glass), air pockets, and fat migration under the casing (National Standard of Iran 2303, 2005).

2.9. Chemical Characteristics

These include moisture, total protein, total fat, total carbohydrates, total phosphorus, pH, salt, residual nitrite, and artificial colors. The test for measuring residual nitrite must be conducted on the fourth day after production or later (Ghaderi *et al.*, 2022; National Standard of Iran 2303, 2005).

2.10. Microbiological Characteristics of Sausages and Salami

These include mold and yeast, *Escherichia coli*, total microbial count, coliforms, Salmonella, Clostridium, and *Staphylococcus aureus* (Kalantari-Hesari *et al.*, 2025; National Standard of Iran 2303, 2005).

2.11. Histological Characteristics

The history of histology dates back to the study of the microscopic structure of tissues and organs and has evolved over the centuries. The science has developed significantly with the development of the microscope and tissue preparation techniques. In the early 17th century, scientists such as Malpighi and Grew studied plant structures using early microscopes and recorded images of plant tissues (National Standard of Iran 6103, 2016; Asadi *et al.*, 2023; Yarvari *et al.*, 2025).

In the 19th century, with the development of the microscope and tissue preparation techniques, histology emerged as an independent science. During this period, scientists such as Johann Purkinje and Marie Villiers developed techniques for staining tissues to

view more detail. Histology also witnessed significant advances in the 20th century. New techniques such as the electron microscope made it possible to observe cellular and molecular structures with very high precision. Histology also became an applied field in medicine and biology with the advent of tissue engineering. And finally, in the 21st century, tissue engineering has made significant advances, with the aim of repairing and replacing damaged tissues using engineered cells and materials. Sausages and salami must be free from unauthorized organs and tissues of livestock and poultry.

2.12. Packaging

Packaging of sausages and salami is done in three ways based on production conditions and product type: conventional (rolled), vacuum-sealed, and modified atmosphere. Permitted synthetic casings such as polyamide and cellulose, as well as natural casings like processed small intestines of halal animals, may be used.

2.13. Important Labeling Notes for Sausages and Salami

- The use of the term “ham” for naming salami is prohibited.
- Storage conditions must be stated (keep refrigerated at 1 to 4 degrees Celsius).
- Production and expiration dates must be included.
- For sausages, the production and expiration dates must be repeated at least three times per meter on each roll. For salami, they must be written at least once per roll.
- Net weight must be stated.
- The name and address of the production unit and the product’s brand name must be included.
- The product type, percentage of meat used, and type of meat must be clearly stated (e.g., “Salami with 40% chicken meat”).
- For products containing chicken paste, the phrase “Contains chicken paste” must be clearly displayed.

This standard makes a brief reference to halal meat animals, and it is recommended that more information be provided about halal meat animals. It also does not provide any recommendations to consumers regarding the harms of excessive consumption. It is recommended that informative annexes be included in the standard text regarding the health risks of excessive consumption of these products.

3. Conclusion

By comparing the national Iranian standards for sausage and salami and their test methods with the standards available in Europe, it can be concluded that most of the existing standards are consistent with European standards. The only difference in the origin of the meat used is that in Europe, the use of some animals such as pigs is permitted, while in Iran only Halal meat can be used. The rest of the microbiological and chemical tests are designed in full accordance with European standards. Regarding the histological test of meat products, no codified standard was found in Europe. (European Natural Sausage Casings Association)

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ORIGINAL RESEARCH PAPER

Prevalence of *Brucella* Contamination in Unpasteurized Dairy Products in Sistan Region, Iran, Using PCR Assay

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Abstract

Brucellosis is one of the most widespread zoonotic diseases globally, posing a significant public health and economic burden. It is primarily transmitted to humans through the consumption of contaminated milk and raw dairy products, or by direct contact with infected animals. This study aimed to determine the prevalence of *Brucella* contamination in unpasteurized dairy products sold in the Sistan region, Iran, utilizing the Polymerase Chain Reaction (PCR) method. A total of 175 unpasteurized traditional dairy samples were collected from various distribution centers in Zabol city. These samples included 35 samples each of milk, yogurt, cheese, buttermilk, and ice cream. DNA extraction was performed using the boiling method. Following DNA extraction, PCR was conducted to detect the presence of *Brucella* bacteria and identify the species, specifically targeting *Brucella abortus* and *Brucella melitensis*. The results revealed that only one sample (0.58% of 175 tested samples) was positive for *Brucella*. This positive sample was identified as *Brucella abortus*, and it originated from one of the 35 milk samples analyzed, indicating a prevalence of 2.86% in unpasteurized milk samples. This low prevalence of *Brucella* infection in the Sistan region, compared to findings from other past studies in the area, is likely attributable to the recent, systematic, and annual free vaccination campaigns against brucellosis in livestock throughout the Sistan region. These findings highlight the effectiveness of ongoing vaccination efforts in reducing the spread of brucellosis in the region.

1. Introduction

Brucellosis, also known as Malta fever or undulant fever, is classified by the World Health Organization (WHO) as one of the seven neglected zoonotic diseases (Hull and Schumaker 2018). This bacterial infection significantly impacts public health and animal production globally, particularly in developing coun-

tries where it perpetuates poverty. While many developed nations have successfully eradicated this important zoonosis (Ning *et al.*, 2018), brucellosis remains widespread in regions such as the Mediterranean, Middle East, Central Asia, and parts of Latin America, making it a global health concern (Maryam *et al.*, 2025; Zhi-guo *et al.*, 2023; Qureshi *et al.*, 2023). It is estimated that 1.6–2.1 million new human cases occur

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annually worldwide, with infected animals and their products being the most probable sources of infection (Qureshi *et al.*, 2023).

Brucella spp. are primarily transmitted to humans through the consumption of unpasteurized dairy products or by direct contact with infected animals or their secretions (Khurana *et al.*, 2020). Among the various species, *Brucella melitensis* and *B. abortus* are the most frequently reported in human and animal infections in Iran, where they pose major public health and economic challenges (Maryam and Saeid 2017). *B. melitensis*, typically associated with sheep and goats, is considered the most virulent species in humans, while *B. abortus* is mainly linked to cattle. Infections in humans often present with nonspecific symptoms such as fever, fatigue, and joint pain, making clinical diagnosis difficult without laboratory confirmation (Bin *et al.*, 2025; Lai, Qiulan, and Zhongjie 2021).

The Sistan region, located in southeastern Iran, is particularly vulnerable to brucellosis due to its high reliance on traditional, unpasteurized dairy production and close contact between livestock and humans. Although national vaccination campaigns have been implemented in recent years to control the disease, sporadic human and animal cases are still reported (Narouei *et al.*, 2022; Ahmadi *et al.*, 2021). These observations highlight the importance of continuous epidemiological surveillance and the need for reliable diagnostic tools to monitor infection sources in high-risk areas.

In this context, the present study aims to evaluate the prevalence of *Brucella* contamination in unpasteurized dairy products collected from the Sistan region using the Polymerase Chain Reaction (PCR) technique. PCR is recognized as a highly sensitive and specific molecular method for the detection of *Brucella* DNA, allowing not only for rapid identification but also for species-level differentiation (Moussa *et al.*, 2011). The findings of this study may provide insight into the effectiveness of ongoing vaccination programs and inform public health strategies to reduce the burden of brucellosis in endemic areas.

2. Materials and Methods

2.1. Study Area and Sample Population

This study was conducted in the Sistan region, focusing on dairy products distributed in Zabol city. The target population included unpasteurized dairy products sold by local vendors, and sampling was conducted in 2023.

2.2. Sample Collection and Storage

A total of 175 samples of traditional unpasteurized dairy products were collected from various distribution centers across Zabol city. The samples included

milk, yogurt, cheese, buttermilk, and ice cream, with 35 samples collected for each product type. Samples were collected using 15 ml Falcon tubes and immediately transported to the laboratory of the Faculty of Veterinary Medicine, University of Zabol, in a cold box containing ice. All equipment, including ampoules, tips (yellow, blue, white), and Eppendorf tubes, were sterilized by autoclaving at 121°C and 1.5 atmospheres for 15 minutes before use. Upon arrival at the laboratory, samples were stored at -20°C until DNA extraction.

2.3. DNA Extraction

DNA extraction from all samples was performed using the boiling method. Initially, 1 ml of each sample was transferred into 1.5 ml Eppendorf tubes and centrifuged at 3500 rpm for 5 minutes. After centrifugation, the supernatant was discarded and replaced with 1 ml of sterile distilled water. The samples were centrifuged again under the same conditions, and the resulting supernatant was removed. Subsequently, 100 µl of sterile distilled water was added to each tube, and the contents were mixed thoroughly using a vortex mixer. The tubes were then incubated in a thermocycler at 98°C for 10 minutes to facilitate DNA release. After incubation, the samples were centrifuged at 14,000 rpm for 10 minutes, and the supernatant containing the extracted DNA was carefully collected using a micropipette. The extracted DNA was stored at -18°C until further analysis by PCR.

2.4. PCR Assay

2.4.1. Primer Synthesis and Preparation

Previously validated primer sets were employed for the detection of *Brucella* species. The synthesis of primers was carried out by Pishgam Biotech Company. For the initial genus-level screening, the *bcs*p31 gene was targeted using the primers B4 (5'-TGG CTC GGT TGC CAA TAA CAA-3') and B5 (5'-CGC GCT TGC CTT TCA GGT CTG-3'), which produced an amplicon of 223 base pairs (Baily *et al.*, 1992). To identify *Brucella abortus* (biovars 1, 2, and 4) and *Brucella melitensis* at the species level, multiplex PCR was performed. For the amplification of *B. abortus*, the primers Ba-SP (5'-GAC GAA CGG AAT TTT TCC AAT CCC-3') and IS711SP (5'-TGC CGA TCA CTT AAG GGC CTT CAT-3') were used, generating a 498 bp fragment. Detection of *B. melitensis* was achieved using the Bm-SP primer (5'-AAA TCG CGT CCT TGC TGG TCT GA-3') in combination with IS711SP, resulting in a 731 bp amplicon (Bricker and Halling 1994).

The primers were reconstituted from lyophilized form following the manufacturer's protocol by dissolving them in sterile deionized water to obtain a stock concentration of 100 pmol/µl. Working solutions with a final concentration of 10 pmol/l were prepared for

routine use and stored at -20°C for long-term preservation.

The final PCR reaction mixture had a total volume of $25\text{ }\mu\text{l}$ and consisted of $12.5\text{ }\mu\text{l}$ of PCR master mix, $9.5\text{ }\mu\text{l}$ of deionized water, $1\text{ }\mu\text{l}$ each of forward and reverse primers, and $1\text{ }\mu\text{l}$ of DNA template.

2.4.2. PCR Cycling Conditions

The thermal cycling protocol used for amplification of the bcs31 gene as well as for species-specific detection of *Brucella abortus* and *Brucella melitensis* followed standardized conditions. The PCR program began with an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 1.5 minutes, annealing at 56°C for 2 minutes, and extension at 72°C for 2 minutes. A final extension step was carried out at 72°C for 5 minutes to complete the reaction.

2.5. Gel Electrophoresis and Visualization

After PCR amplification, the products were confirmed using horizontal electrophoresis on a 2% agarose gel. A 100bp-3kbp DNA ladder was used as a size marker. $5\text{ }\mu\text{l}$ of each PCR product was loaded onto the gel. The gel was stained with ethidium bromide and visualized under UV light using a Gel Documentation system. Ethidium bromide is a carcinogen, so appropriate safety precautions (gloves, mask, and lab coat) were observed.

2.6. Data Analysis

The prevalence of *Brucella* contamination in the sample population was calculated using a binomial distribution with 95% confidence intervals.

3. Results

Out of the 175 unpasteurized dairy samples examined in this study, only one sample (0.58%) (With 95% CI from 0.01% to 3.1%) tested positive for *Brucella*. This positive sample was specifically milk, and the identified species was *Brucella abortus*. This translates to a prevalence of 2.86% within the 35 milk samples analyzed.

The positive results were confirmed by the presence of specific DNA bands for the *Brucella* genus and *Brucella abortus* species using the respective primer sets.

This observed prevalence of *Brucella* infection in unpasteurized dairy products in the Sistan region is significantly lower compared to historical data and other studies conducted in the past years in the same region. For instance, a 2018 study detected *Brucella melitensis* in 35% of aborted sheep fetuses in the Sistan region. Another study in Sistan reported a seroprevalence of

2.3% for *Brucella* in sheep and goats, with PCR showing a 17% positivity rate in 2021.

The current low prevalence is strongly attributed to the sustained and systematic free brucellosis vaccination programs for livestock that have been implemented annually in the Sistan region in recent years.

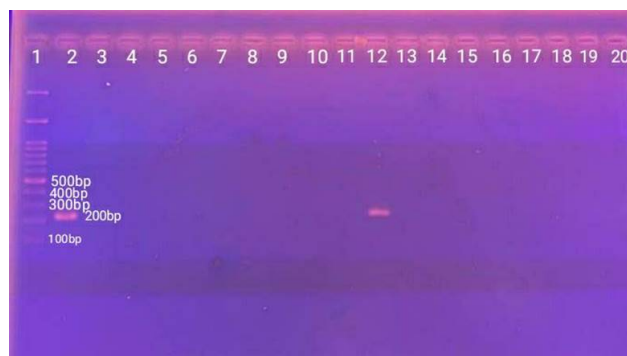


Fig. 1. Electrophoresis of PCR Products (bcs31 gene) showing *Brucella* genus amplification (This image would show a band at 223 bp for the positive sample, indicating the presence of *Brucella* DNA)



Fig. 2. Electrophoresis of PCR Products (Ba-SP and IS711 primers) showing *Brucella abortus* species amplification (This image would show a band at 498 bp for the positive sample, confirming *Brucella abortus*)

4. Discussion

The findings of this study, revealing a very low prevalence of *Brucella* contamination (0.58%) in unpasteurized dairy products in the Sistan region, and specifically *Brucella abortus* in only one milk sample, are noteworthy and encouraging. This outcome stands in stark contrast to the higher prevalence rates reported in historical studies within Iran and internationally.

Previous research in Iran has consistently shown higher rates of brucellosis. For example, Jawadi *et al.*, reported a seroprevalence of 4.53% in sheep and 5.33% in goats in East Azerbaijan province (Javadi *et al.*, 2007). Kaboutari *et al.* found a 6.4% seroprevalence in sheep and goats and a 25.3% flock level prevalence in southern Kerman province (Kaboutari *et al.*,

2015). Mohammadi et al. detected *Brucella melitensis* in 17.5% of aborted sheep fetuses in Gonbad-e Qabus (Mohammadi et al., 2019). In Zabol itself, a 2016 study found that 7% of cow milk samples tested positive for *Brucella abortus* and *Brucella melitensis*, with 5% being *B. abortus* positive and 2% *B. melitensis* positive (Ahmadi et al., 2021). Even in a 2017 study specifically on sheep and goats in the Sistan region, a PCR-based prevalence of 11.3% for *Brucella* was reported, with all positive samples being *B. melitensis*. These comparisons underscore the significant reduction observed in the current study (Narouei et al., 2022; Ahmadi et al., 2021).

One of the most important factors contributing to the reduced prevalence of brucellosis observed in the present study appears to be the implementation of regular phases of free vaccination campaigns against brucellosis in livestock across the Sistan region in recent years. These government-supported programs, which impose no financial burden on animal owners, have likely played a key role in controlling the disease. Vaccination of animals is globally recognized as one of the most effective tools for preventing human brucellosis. Countries like Kuwait have demonstrated the efficacy of extensive animal vaccination programs in controlling brucellosis in cattle, sheep, and goats (Al-Khalaf, Mohamad, and Nicoletti 1992). Control strategies built upon vaccination, testing, and culling have proven successful in many developed countries (Ning et al., 2018).

B. melitensis is typically the most prevalent species in sheep and goats and the primary cause of human brucellosis. Compared to other *Brucella* species, *B. melitensis* causes the most severe and acute form of the disease in humans and is considered the most pathogenic for humans (Patrycja et al., 2018).

Brucella abortus is primarily associated with brucellosis in cattle. It is known to cause reproductive disorders such as abortion, infertility, and decreased milk production in infected animals. Although it is less pathogenic to humans compared to *Brucella melitensis*, it remains a zoonotic agent of concern, particularly in regions where cattle are raised alongside other ruminants (Dereje 2022).

In the present study, the detection of *B. abortus* in a milk sample—despite the predominance of sheep and goat dairy production in the region—suggests a possible bovine origin of contamination. This unexpected finding highlights the need for sustained surveillance not only in small ruminants but also in cattle, even in areas where bovine dairy farming is limited. It also emphasizes the risk of cross-species transmission and the importance of implementing comprehensive brucellosis control strategies that encompass all livestock

The study also reinforces the utility of PCR as a highly sensitive and specific diagnostic tool for *Brucella* detection, especially when the bacterial load in samples might be low. Unlike traditional serological

methods that detect antibodies and can yield false positives due to cross-reactions or persistent antibodies post-treatment, PCR directly identifies bacterial DNA, making it reliable for diagnosing active infections and even low bacterial counts. This is particularly advantageous for milk samples where the presence of live *Brucella* poses a direct risk of transmission (Batinou et al., 2022; Ahmadi et al., 2021).

5. Conclusion

Despite the positive trend, the presence of even a single *Brucella* positive sample in unpasteurized dairy products underscores the ongoing risk to public health, especially given the common practice of consuming raw dairy in the region. The “One Health” approach, integrating animal health and public health efforts, is crucial for sustained success in brucellosis control.

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ORIGINAL RESEARCH PAPER

Comparative Histomorphologic ,Histomorphometric and Histochemistry Studies of Cecum in Adult Male and Female Black Local Turkeys of West Azarbaijan-Iran

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Abstract

Domestic fowl, including chickens, turkeys, and ducks, possess an organ known as the cecum, crucial for digestion and disease resistance due to its significant lymphoid tissue existence. However, limited information exists on the histology and histochemistry of the cecum in native breeds like the Azerbaijani Black Turkeys. This research delves into the histological examination of the cecal regions in male and female turkeys. For this purpose, to study the histology of the turkey cecum, 5 male and 5 female turkeys with an approximate weight of 4 to 6 kg were selected. After anesthetizing and euthanizing the animals, their ceca were separated and placed in a 10% formalin buffer solution. Tissue sections were prepared through routine paraffin embedding technique and stained with hematoxylin-eosin for general examination and histomorphometry. Moreover, PAS staining was used to evaluate the dispersion of goblet cells in three parts of the male and female turkey cecum, and Masson's trichrome staining was applied for evaluating collagen fibers. Results indicated anatomical similarities in cecal positioning among birds, with males showing greater mucosal layer thickness and lymph node size compared to females. Moreover, lymph node density varied between genders, with females exhibiting higher cell counts. This study provides valuable insights into the cecal structure of turkeys, shedding light on gender-specific histological differences and lymphoid cell distribution.

1. Introduction

The turkey (*Meleagris gallopavo*) is a species of bird belonging to the order Galliformes. It is classified under the genus *Meleagris* and is native to regions of Latin America. Turkeys are characterized by a fleshy wattle hanging below their beak and a distinctive fleshy protuberance (snood) extending from the top of their beak, distinguishing them from other poultry species (Fathi *et al.*, 2009). Like many other species within the order Galliformes, male turkeys are larger and more vi-

brantly colored than females. Turkeys, like other birds, have different breeds; some are wild and cannot be domesticated, whereas others are native breeds reared for meat and egg production (Fathi *et al.*, 2009).

The cecum plays a pivotal role in the digestive and immune systems of domestic fowl, including chickens, turkeys, and ducks, by facilitating nutrient absorption, microbial fermentation, and disease resistance (Erf, 2004, Turk, 1982). Despite its importance, native breeds of poultry, such as the Azerbaijani Black Turkey, have been the subject of limited histological

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and histochemical studies. Investigating the structural and cellular composition of the cecum in these birds can enhance our understanding of their digestive efficiency and immunological adaptations.

The avian digestive tract is structured to optimize nutrient utilization, with the cecum functioning as a key organ for cellulose digestion, water absorption, and immune surveillance (Zaher *et al.*, 2012). Previous research has highlighted the presence of extensive lymphoid tissue within the cecum, including cecal tonsils, which contribute to pathogen defense and mucosal immunity (Rezaian and Hamed, 2006). Additionally, the structural organization of the cecum, including mucosal folds, Lieberkühn glands, and goblet cells, plays a crucial role in maintaining intestinal homeostasis (Getty *et al.*, 1975).

While numerous studies have examined the digestive morphology of commercial poultry species, few have focused on native turkey breeds, particularly those reared in Azerbaijan. Given their significant role in local poultry farming, understanding their digestive histology and immune function is essential for optimizing breeding programs and improving poultry health (Erf, 2004, Firth, 1977). The comparison between male and female turkeys may reveal gender-specific anatomical differences, particularly in terms of mucosal structure, lymphoid tissue distribution, and collagen fiber organization (Ciriaco *et al.*, 2003).

This study aims to conduct a detailed histological examination of the cecal regions of male and female turkeys, utilizing advanced staining techniques such as hematoxylin-eosin for general tissue analysis, PAS for goblet cell detection, and Masson's trichrome for collagen fiber evaluation. Through these analyses, the thickness of the mucosal layer, cecal villi height, and lymphoid tissue dispersion will be compared across different sections of the cecum (proximal, middle, and distal).

Ultimately, this research seeks to enhance our understanding of digestive physiology in native turkeys, bridging the existing gap in histological studies and providing valuable insights into avian immunology and digestion. By examining gender-specific differences in histomorphometry, these findings may contribute to future advancements in poultry health management and nutritional research.

2. Materials and Methods

2.1. Sample Collection and Preparation

This study was conducted on ten adult turkeys (five males and five females), approximately one-year-old, naturally fed, and belonging to the local ecotype of West Azerbaijan, Iran (commonly known as Black Turkeys). Following weighing and humane euthanasia, cecal tissue samples were collected from three anatomical regions: proximal, middle, and distal parts. The samples were immediately fixed in a 10% buffered formalin solution to preserve tissue integrity.

ical regions: proximal, middle, and distal parts. The samples were immediately fixed in a 10% buffered formalin solution to preserve tissue integrity.

3. Histological Study of the Cecum

Given that the cecum in turkeys is bilaterally structured, tissue samples were obtained from three regions of each cecal section-proximal, middle, and distal. The samples were fixed in a 10% buffered formalin solution for proper tissue preservation. Following fixation, tissue specimens were processed, embedded in paraffin, and prepared for histological examination.

4. Histological Preparation and Analysis

For histological evaluation, the samples were stained using the hematoxylin-eosin (H&E) method, allowing for general tissue morphology and histomorphometric analysis. Additional staining protocols were employed to highlight specific cellular structures.

Periodic Acid-Schiff (PAS) staining was used to detect carbohydrate containing structures and goblet cell distribution.

Masson's Trichrome staining was performed to assess collagen fiber distribution.

5. Histomorphometry

Cell counts and structural assessments were conducted using a binocular light microscope equipped with calibrated grid eyepieces, with ten microscopic fields per region (proximal, middle, distal) were randomly selected for each sample. Goblet cells were counted in 0.04 mm areas under 40X.

Quantitative histomorphometric analysis was conducted using hematoxylin-eosin stained sections to measure following parameters in proximal, middle, distal parts:

Cecal mucosal layer thickness, Cecal fold height, Cecal villi height, Lieberkühn gland height, Muscular layer thickness, Dense lymphoid tissue distribution, Lymphoid nodule diameter, Lymphocyte density in dispersed and dense lymphoid tissue, Goblet cell distribution (PAS-stained sections) and Collagen fiber distribution (Masson's Trichrome-stained sections).

6. Statistical Analysis

Quantitative data were analyzed using ImageJ software (US National Institutes of Health), and results were expressed as Mean $\pm$ SEM. Normality and variance homogeneity were evaluated using the Kolmogorov-Smirnov test, followed by one-way ANOVA and Duncan's multiple range test for comparative analysis

(SPSS, version 18). Statistical significance was determined at $P < 0.05$.

7. Result

7.1. Clinical Observations and Body Weight Changes

In both male and female turkeys, the cecum appeared as a paired organ, located at the junction of the ileum and rectum. It consisted of three distinct regions-proximal, middle, and distal with unequal sizes. The average body weights of male and female turkeys are presented in Fig. 1.

8. Histological Findings

Histological analysis of the proximal cecal region showed thick mucosal folds with numerous structures. Villi were present on the folds and interfold spaces, displaying variable lengths. Some villi were finger-like, while others were sharper or absent in specific areas. Lieberkuhn glands were uniformly distributed, and

lymphoid tissue appeared both as diffuse and nodular aggregates. The epithelial layer consisted of simple columnar cells, with goblet cells and basal cells clearly identifiable (Fig. 2(a), 2(b), 2(c), Fig. 3).

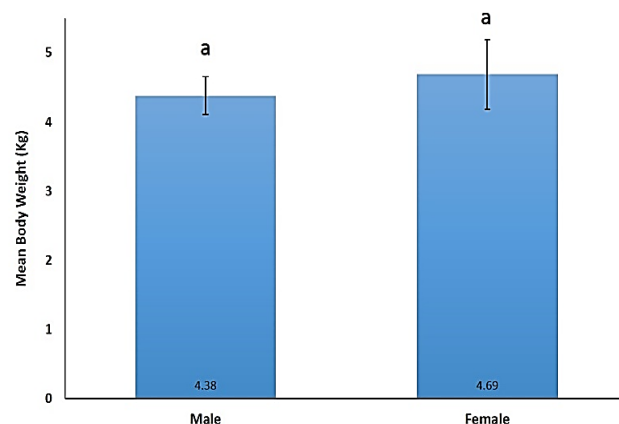


Fig. 1. The average live weight of turkeys in kilograms. Similar superscript denotes there is no significant differences ($P > 0.05$) in body weights between male and female turkeys.

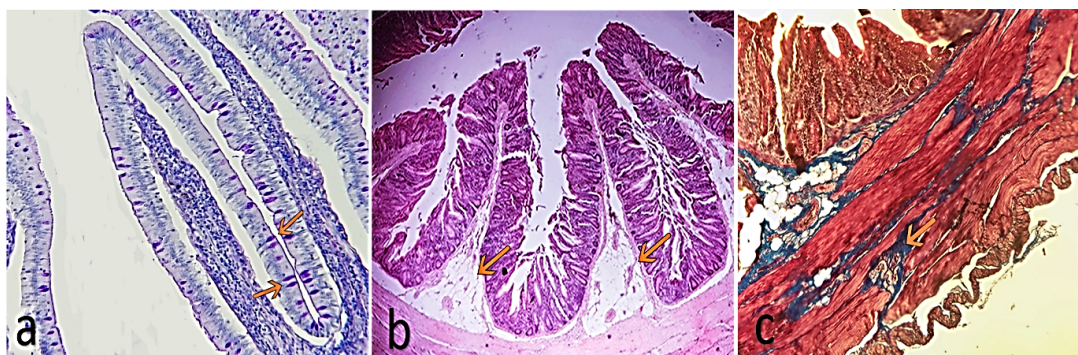


Fig. 2. a) Distribution of goblet cells (arrowhead) within the epithelium of the proximal cecal section in male turkeys. (X40) Periodic Acid-Schiff (PAS) staining. b) Cecal folds and accumulation of adipose tissue within the submucosa of the proximal cecal section in male turkeys. H&E, X20. c) Neural network of Auerbach's plexus (arrow) distributed within the muscular layer of the proximal cecal section in male turkeys. Masson's Trichrome, X40.

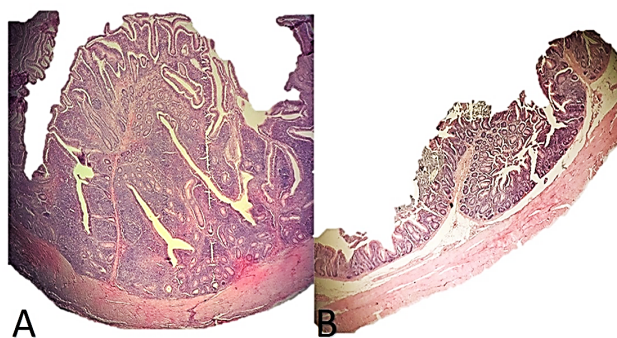


Fig. 3. Variation in the distribution of cecal folds and villi within the proximal section of the male turkey cecum. A) Presence of large folds and prominent villi. B) Smaller folds and reduced villi. H&E, 20X.

In the middle section, folds were dense and elongated, often containing adipose tissue at the base. Villi structures were less frequent and shorter, with reduced goblet cell density. Lieberkuhn glands appeared dilated, while lymphoid tissue remained diffusely scattered throughout the lamina propria (Fig. 4).

The distal cecal region exhibited fewer mucosal folds but longer, thinner villi. The epithelium showed a high density of goblet cells, especially within the Lieberkuhn glands. Lymphoid tissue was less concentrated, appearing predominantly as diffuse aggregates. Some specimens displayed shortened folds with compact villi, contrasting with the proximal and middle sections (Fig. 5).

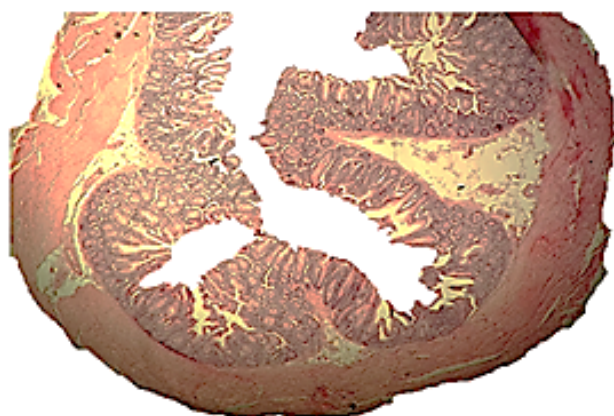


Fig. 4. Dense mucosal folds with extended length observed in the middle section of the male turkey cecum, accompanied by the accumulation of adipose connective tissue at the base of the folds. Nodular lymphoid tissue is absent in this section. H&E, 20X.

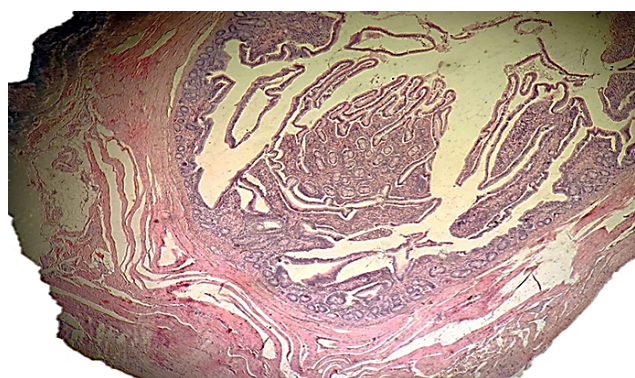


Fig. 5. Dense and elongated villi observed in the distal section of the male turkey cecum. Nodular lymphoid tissue is not seen in this region. H&E, 20X.

9. Histological Findings in Female Turkey Cecum

Microscopic inspection of the proximal cecum in female turkeys revealed two distinct types of villi. In some sections, thin and elongated villi extended deep

into the cecal lumen, while in others, villous structures appeared shorter and thicker. Lymphoid tissue in the lamina propria was observed both as nodular clusters and diffuse forms, with cecal tonsils clearly visible in some samples (Fig. 6(P)). The distribution of Lieberkuhn glands varied significantly, with some regions showing dense glandular occurrence, while others had reduced gland disseminations. The muscular layer was composed of an inner circular and outer longitudinal layer, with the inner layer appearing thicker.

In the middle cecal section, mucosal folds became more dominant, while cecal villi were noticeably shorter. Lymphoid tissue density decreased, and the cecal lumen widened. A significant accumulation of fat-rich connective tissue was observed at the base of the folds (Fig. 6(M)).

In the distal cecum, the muscular layer weakened, and mucosal folds became considerably shorter. The submucosa was densely packed with fat, while lymphoid aggregates remained prominent, appearing both as nodular and diffuse clusters. Areas with higher lymphoid concentration exhibited reduced fat deposits, and villous structures appeared sparsely distributed and very short (Fig. 6(D)).

10. Results of histomorphometric assessments

This study examined key morphometric differences across the three cecal sections in male and female turkeys to evaluate structural variations.

11. Cecal Mucosal Thickness

Dimensions of mucosal thickness remained similar in the proximal and middle cecum, but significantly decreased in the distal section in male turkeys (Fig. 7(A)). In female turkeys, mucosal thickness varied between sections, with significant differences between the middle and distal regions, though the proximal segment remained unchanged. Overall, male turkeys exhibited significantly thicker mucosal layers through all the cecal regions in comparison to females (Fig. 7(B)).

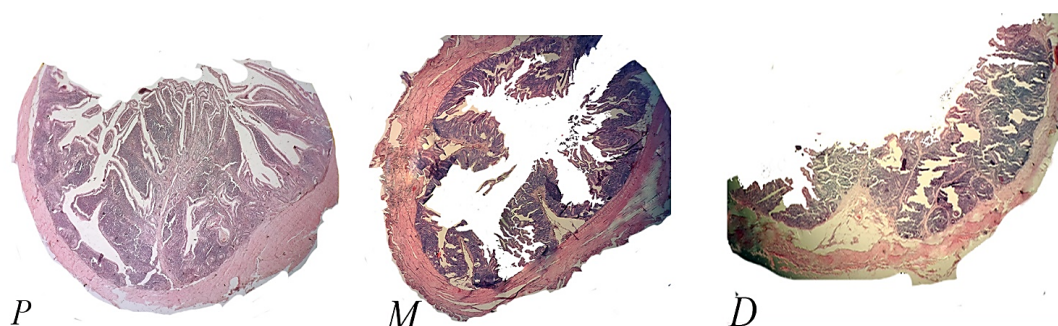


Fig. 6. Distribution of mucosal folds, villi, lymphoid tissue, and adipose tissue in the three sections (proximal (P), middle (M), and distal (D)) of the female turkey cecum. H&E, 20X.

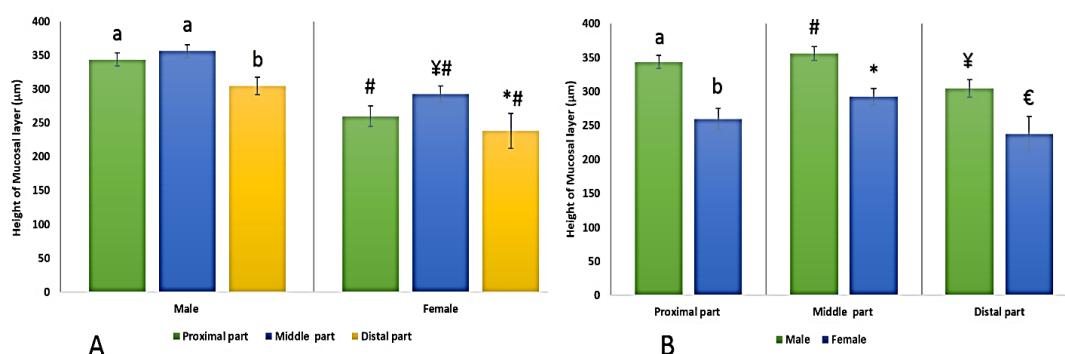


Fig. 7. A) Mean thickness of the mucosal layer across the three cecal sections (proximal, middle, distal) in male and female turkeys. B) Comparison of mucosal layer thickness in the three sections (proximal, middle, distal) between male and female turkeys. Different superscripts within each section indicate statistically significant differences ($P < 0.05$).

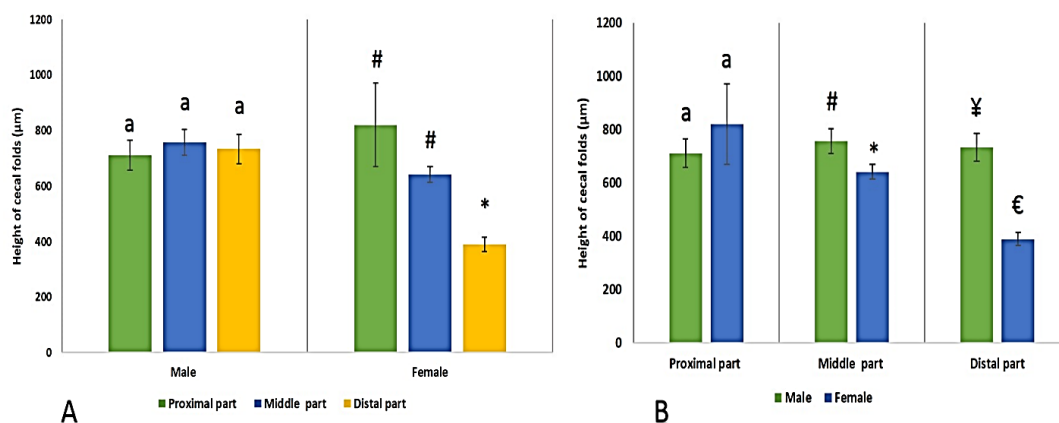


Fig. 8. A) Mean height of cecal folds in the three regions (proximal, middle, distal) of the cecum in male and female turkeys. B) Comparative analysis of cecal fold height in the three regions (proximal, middle, distal) between male and female turkeys. Different superscript letters within each region denote statistically significant differences ($P < 0.05$).

12. Cecal Fold Height

In male turkeys, fold height remained consistent across the three regions, showing no significant differences (Fig. 8(A)). However, in female turkeys, fold height gradually decreased from proximal to distal sections, showing significant variation (Fig. 8(A)). Comparisons between both sexes indicated that males had consistently taller cecal folds, with statistically significant differences in the middle and distal regions (Fig. 8(B)).

13. Cecal Villi Height

The distribution and height of cecal villi varied significantly among male turkeys, with large differences through all three cecal regions. In female turkeys, proximal villi appeared significantly longer, whereas middle and distal villi were markedly shorter, showing significant reductions in height (Fig. 9(A)). Comparisons between both the sexes revealed that, male turkeys

bearing taller villi through all three cecal regions, with statistically significant differences in the proximal and distal regions (Fig. 9(B)).

14. Histological Analysis of Lieberkuhn Glands

The measurement of Lieberkuhn gland height in the three cecal regions (proximal, middle, distal) revealed prominent differences between male and female turkeys. In male turkeys, the proximal and middle regions exhibited similar gland heights, whereas the distal regions showed a significant reduction (Fig. 10(A)). However, in female turkeys, the proximal and distal regions had similar gland sizes, while the middle regions displayed significantly longer glands. Comparative analysis between both sexes indicated that, males had larger Lieberkuhn glands overall, with significant differences in the proximal and distal regions compared to females (Fig. 10(B)).

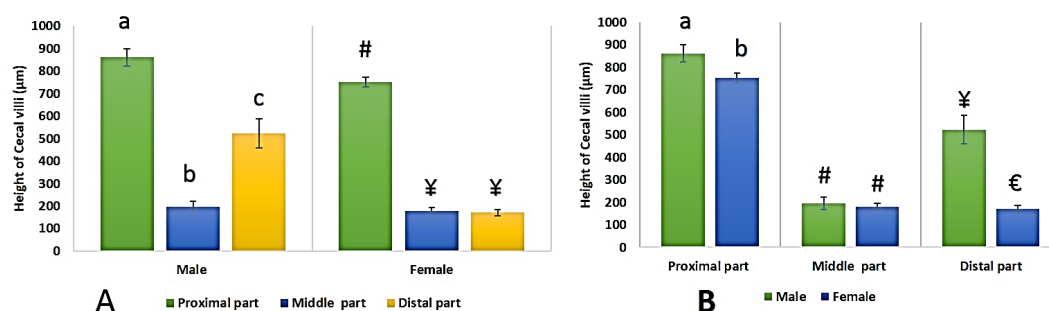


Fig. 9. A) Mean height of cecal villi across the three regions (proximal, middle, distal) of the cecum in male and female turkeys. B) Comparative analysis of cecal villi height in the three regions (proximal, middle, distal) between male and female turkeys. Different superscript letters within each region indicate statistically significant differences ($P<0.05$).

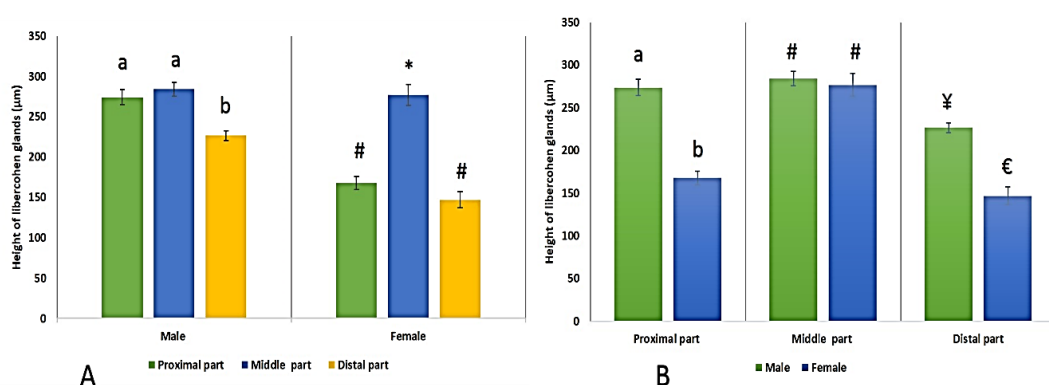


Fig. 10. A) Mean height of Lieberkuhn glands through three regions (proximal, middle, distal) of the cecum in male and female turkeys. B) Comparative analysis of the height of Lieberkuhn glands in the three regions (proximal, middle, distal) between male and female turkeys. Different superscript letters within each section indicate statistically significant differences ($P<0.05$).

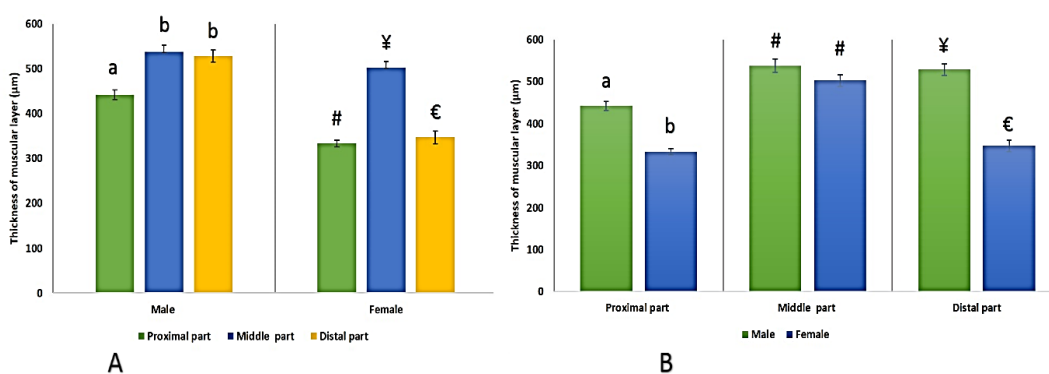


Fig. 11. A) Mean thickness of the muscular layer through the three regions (proximal, middle, distal) of the cecum in male and female turkeys. B) Comparative analysis of muscular layer thickness in the three regions (proximal, middle, distal) between male and female turkeys. Different superscript letters within each section indicate statistically significant differences ($P<0.05$).

15. Muscular Layer Thickness of the Cecum

The thickness of the muscular layer varied between male and female turkeys. In males, muscular thickness increased regularly toward the distal region, although no significant difference was observed between the middle and distal regions (Fig. 11(A)). In con-

trast, female turkeys exhibited the thickest muscular layer in the middle region, with significant thinning in the proximal and distal regions (Fig. 11(A)). Once comparing male and female turkeys, males consistently had thicker muscular layers across all regions, with significant differences in the proximal and distal regions (Fig. 11(B)).

16. Lymphoid Tissue Distribution

The analysis of various lymphoid parameters within the three cecal regions of male and female turkeys, revealed differences in lymphoid nodule distributions as follow:

In male turkeys, nodular lymphoid structures were present only in the proximal section (Table 1).

In female turkeys, nodular lymphoid structures were abundant in both the proximal and distal regions (Table 2.)

Comparative analysis showed that, the females had significantly higher nodular lymphoid density in the proximal section (Table 3).

Further analysis of lymphoid nodule diameter revealed that although males had fewer lymphoid nodules, their nodules were significantly larger compared to those in females (Table 1, 2). Lymphocyte distribution within diffuse and nodular lymphoid tissue was quantified using 40X magnification and a calibrated grid eyepiece (Table 1, 2).

In male turkeys, diffuse lymphoid accumulation was

significantly higher in the proximal region, decreasing in middle and distal regions (Table 1).

In female turkeys, diffuse lymphoid accumulation were most concentrated in the proximal and distal regions, with lower density in the middle region (Table 1).

Comparing both sexes, significant differences in diffuse lymphoid cell accumulation were observed in the proximal and distal regions, whereas the middle region showed not at all prominent variation (Table 3).

17. PAS Staining and Goblet Cell Distribution

PAS-stained sections revealed PAS-positive reactions within goblet cells, indicating intracellular mucin accumulation (Fig. 12). PAS staining also highlighted basal lamina structures surrounding epithelial layers, blood vessels, and smooth muscle fibers, particularly within muscle-separating membranes (endomysium, perimysium, epimysium).

Table 1

Comparison of various parameters of diffuse and nodular lymphoid tissue in different regions of the male turkey cecum.

Region of Cecum	Mean Number of Lymphoid Nodules (N)	Mean Diameter of Lymphoid Nodules (m μ)	Distribution of Lymphocytes in Lymphoid Nodules (0.04 mm ²)	Distribution of Lymphocytes in Diffuse Lymphoid Tissue (0.04 mm ²)
Proximal	8.0 $\pm$ 0.894	361.33 $\pm$ 20.867	1964.26 $\pm$ 19.57	1379.4 $\pm$ 114.4 ^a
Middle	0	0	0	899.11 $\pm$ 184.9 ^b
Distal	0	0	0	855.22 $\pm$ 154.3 ^b

Table 2

Comparison of various parameters of diffuse and nodular lymphoid tissue in different regions of the female turkey cecum.

Region of Cecum	Mean Number of Lymphoid Nodules (N)	Mean Diameter of Lymphoid Nodules (μ)	Distribution of Lymphocytes in Lymphoid Nodules (0.04 mm ²)	Distribution of Lymphocytes in Diffuse Lymphoid Tissue (0.04 mm ²)
Proximal Section	22.0 $\pm$ 0.57 ^a	260.5 $\pm$ 44.561 ^a	1699.52 $\pm$ 105.23 ^a	1061.5 $\pm$ 107.6 ^a
Middle Section	0	0	0	885.32 $\pm$ 116.6 ^{ab}
Distal Section	8.33 $\pm$ 1.55 ^b	195.64 $\pm$ 28.229 ^a	2044.45 $\pm$ 71.11 ^b	1292.2 $\pm$ 209.3 ^a

Dissimilar superscripts within each column indicate statistically significant differences (P < 0.05).

Table 3

Comparative Analysis of Various Parameters of Diffuse and Nodular Lymphatic Tissue in Different Cecal Regions between Male and Female Turkeys.

Male/Female	Mean Number of Lymphoid Nodules (N)	Mean Diameter of Lymphoid Nodules (μ m)	Distribution of Lymphocytes in Lymphoid Nodules (0.04 mm ²)	Distribution of Lymphocytes in Diffuse Lymphoid Tissue (0.04 mm ²)
Proximal Male	0.8 $\pm$ 0.894 [#]	361.33 $\pm$ 867/20 [#]	1964.26 $\pm$ 19.57 [#]	1379.4 $\pm$ 114.4 ^a
Proximal Female	0.22 $\pm$ 0.57 [*]	260.5 $\pm$ 44.561 [*]	1699.52 $\pm$ 105.23 [#]	1061.5 $\pm$ 107.6 ^b
Middle Male	0	0	0	899.11 $\pm$ 184.9 [#]
Middle Female	0	0	0	855.32 $\pm$ 116.6 [#]
Distal Male	0	0	0	855.22 $\pm$ 154.3 [¥]
Distal Female	8.33 $\pm$ 1.55	195.64 $\pm$ 28.229	2044.45 $\pm$ 71.11	1292.2 $\pm$ 209.3

Dissimilar superscripts within each column indicate statistically significant differences (P < 0.05).

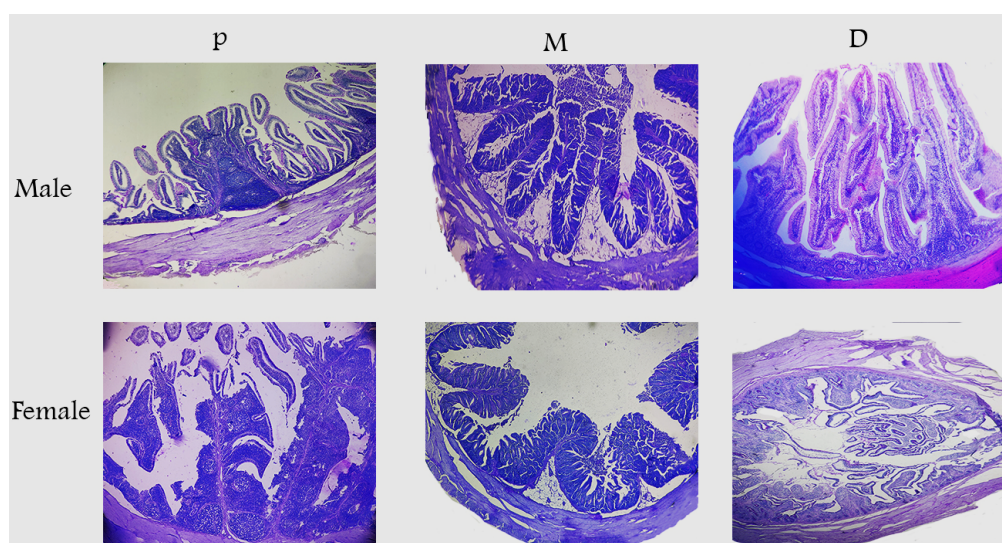


Fig. 12. Distribution of tissues with PAS-positive reactions in the three regions of the cecum: proximal (P), middle (M), and distal (D) in male and female turkeys. PAS, 10X.

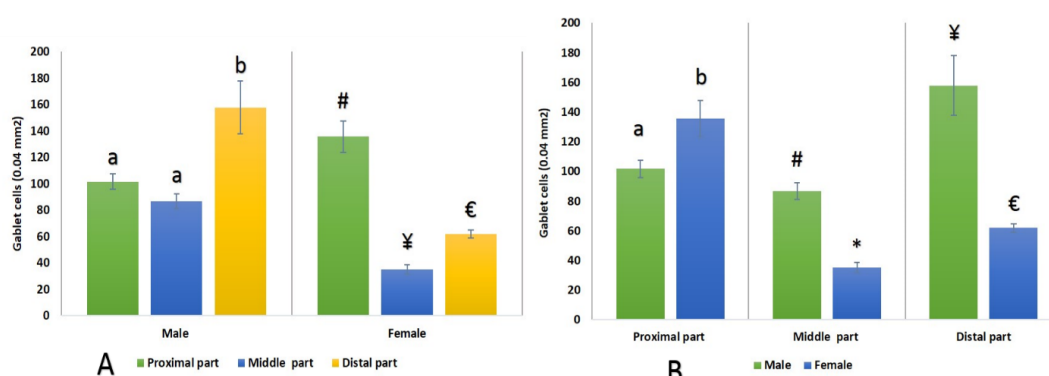


Fig. 13. A) Mean distribution of goblet cells in the three regions (proximal, middle, distal) of the cecum in male and female turkeys in 0.04 mm². B) Comparison of the mean distribution of goblet cells in the three regions (proximal, middle, distal) of the cecum between male and female turkeys in 0.04 mm². Different superscripts in each region indicate a significant difference ($p < 0.05$).

18. Quantification of goblet cell distribution revealed an inconsistent pattern across cecal regions as follow

In male turkeys, goblet cell distribution increased progressively toward the distal region of the cecum (Fig. 13(A)).

In female turkeys, goblet cell distribution was highest in the proximal region of the cecum (Fig. 13(A)).

19. Comparative analysis showed significant differences between genders as follow

In the proximal region of the cecum, males had more goblet cells than females (Fig. 13(B)).

In the middle and distal regions of the cecum, females had higher goblet cell counts than males (Fig. 13(B)).

20. Masson's Trichrome Staining Findings

Masson's Trichrome staining was used to evaluate collagen fiber distribution within the cecal tissue. In this staining, collagen fibers appeared blue, as a clearly distinguishing connective tissue structures. Typical stained sections are showed in Fig. 14.

Regions with dense fibrous connective tissue showed strong blue staining, indicating a higher collagen occurrence. However, areas where connective tissue was intermixed with nodular and diffuse lymphoid aggregates or adipose tissue exhibited lower collagen density, resulting in less intense blue pattern. Additionally, blue staining was observed along the basal lamina of blood vessels, connective tissue surrounding neural ganglia, and muscle fibers, further highlighting collagen distribution in the cecal architecture. Collagen occurrence was also prominent in the serous layer.

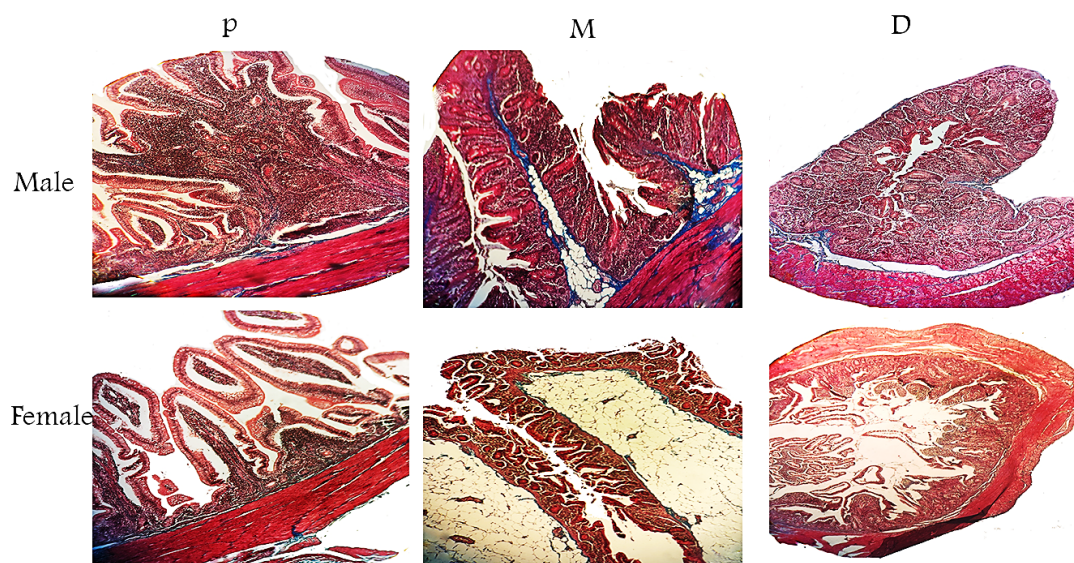


Fig. 14. collagen fibers distribution in three regions of the cecum: proximal (P), middle (M), and distal (D) in male and female turkeys. Masson Trichrome, 20X.

21. Quantitative Analysis of Collagen Fiber Distribution

Collagen fiber dispersion was examined in three anatomical regions (proximal, middle, distal) of the cecum in male and female turkeys using Masson's Trichrome-stained sections and Image J software for digital analysis. Statistical comparison revealed no significant differences in collagen fiber distribution between males and females through cecal regions (Fig. 15).

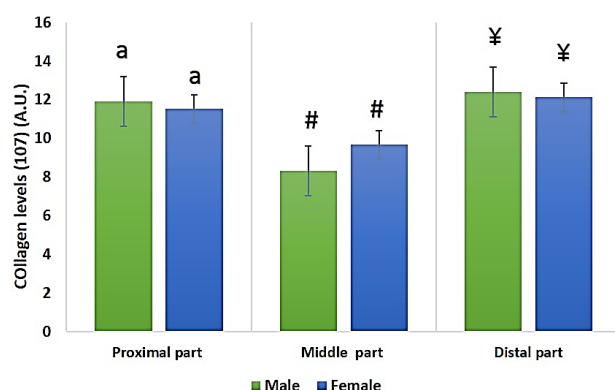


Fig. 15. Comparison of collagen fiber distribution in the three regions (proximal, middle, distal) of the cecum between male and female turkeys. Different superscripts in each region indicate a significant difference ($p < 0.05$).

22. Discussion

The turkey (*Meleagris gallopavo*) belongs to Galliformes, and similar to other galliform birds, males

exhibit larger body size and distinct anatomical features compared to females. Despite extensive studies on commercial poultry breeds, research on native turkeys, particularly the Azerbaijani Black Turkey, is scarce. This study aimed to bridge that gap by investigating histological and histomorphometric differences between sexes in the cecum.

The paired ceca, essential for nutrient absorption and immune regulation, are present in most galliform birds but absent in certain avian groups, such as parrots, woodpeckers, owls, hummingbirds, and falcons (Bezuidenhout, 1993, Hassan and Moussa, 2012, Pourhaji and Hashemi, 2019). Findings confirmed that Azerbaijani Black Turkeys possess paired ceca, structurally consistent with other poultry species, situated at the ileocecal junction and divided into three anatomical regions including proximal, middle, and distal.

Histological examination demonstrated structural variations across cecal regions, holding digestive competence and microbial activity. The presence of mucosal folds and villi enhances fermentation of complex carbohydrates, notably cellulose and hemicellulose, similar to observations in ostriches (Bezuidenhout, 1993). According to results of this study, in male turkeys, thick mucosal folds with abundant villi were identified, whereas in females, fold height decreased progressively toward the distal section. Moreover, males exhibited significantly thicker mucosal layers, reinforcing the hypothesis that sex-related anatomical differences influence digestive function.

Beyond digestion, the avian cecum plays a major role in immune defense. It serves two primary functions: Microbial fermentation, generating short-chain fatty acids (acetic acid, butyric acid, propionic acid) for gut health, and Immune surveillance, supported by

cecal tonsils and lymphoid aggregations (Hussein and Reshag, 2019).

Lymphoid structures within the cecum exhibit species-specific differences. In chickens, cecal tonsils are highly structured, whereas in kiwi birds, they are absent (Potter *et al.*, 2006). In ostriches, lymphoid nodules are densely packed within the proximal cecum, whereas in geese and ducks, they are less prominent (Warui *et al.*, 2009). According to the findings of this study, recommendation of sex-related variations in lymphoid tissue distribution in Azerbaijani Black Turkeys is sound, as in male turkeys there were fewer lymphoid nodules, nevertheless their nodules were significantly larger, suggesting a localized immune defense complex; but female turkeys exhibited greater nodule density, particularly in the distal section, a pattern not previously reported in other avian species. This proposes that, female turkeys may depend on enhanced immune activity in distal cecal regions, potentially due to higher pathogen exposure in the lower digestive tract.

PAS staining confirmed the presence of goblet cells, essential for mucin production and intestinal barrier maintenance (Al-Agele *et al.*, 2020). Our findings indicated sex-specific differences in goblet cell dispersal. In male turkeys, goblet cell records increased toward the distal section, suggesting progressive mucin secretion. In female turkeys, goblet cell records were highest in the proximal cecum, potentially reflecting local protective adaptation. Males exhibited more goblet cells in the proximal region, whereas females had greater goblet cell counts in the middle and distal regions.

Masson's Trichrome staining highlighted collagen fiber presence, notably around blood vessels, neural ganglia, and muscle layers, reinforcing structural stability within the cecum. However, no significant differences in collagen fiber distributions were observed between male and female turkeys, suggesting similar connective tissue organization exists between genders.

23. Conclusion

This study provides important insights into the histomorphologic and histomorphometric deviations in male and female Azerbaijanian black turkeys, particularly concerning lymphoid tissue disseminations, mucosal thickness, and digestive tract morphology. The findings highlight sex-related differences in immune constructions and digestive adaptations, emphasizing the need for further research into their physiological relevance.

By integrating advanced histological and molecular techniques, future research can provide a comprehensive perspective on avian digestion and immunity, contributing to improvements in poultry breeding science, health management, and nutritional optimization.

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ORIGINAL RESEARCH PAPER

Histological and Morphometric Characterization of Major Salivary Glands in Iranian Long-eared Hedgehog (*Hemiechinus Auritus*)

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Abstract

The Iranian long-eared hedgehog (*Hemiechinus auritus*) represents a unique model of mammalian adaptation to arid environments. Despite the critical role of salivary glands in maintaining oral homeostasis and facilitating survival in water-scarce conditions, no histological studies have been conducted on these structures in this species. This pioneering investigation aimed to provide the first comprehensive histological description and morphometric analysis of the three major salivary glands in *H. auritus*. In this study, five deceased adult hedgehogs (3 females, 2 males) were utilized. Following standard necropsy procedures, parotid, submandibular, and sublingual glands were harvested and fixed in 10% buffered formalin. Tissues underwent routine histological processing including dehydration through ascending alcohol series, clearing with xylene, and paraffin embedding. Serial sections (5-7 μm) were stained with hematoxylin-eosin. Digital image analysis system comprising light microscope (Medic M-107 BN, China), camera (Dino-Lite), imaging software (Dino Capture V.2), and analysis software (Image-Pro Plus v.6) was employed to determine serous/mucous acinar ratios. This descriptive study focused solely on reporting tissue architecture without statistical comparisons. The parotid gland exhibited exclusively serous architecture (100%) with densely packed acini, prominent striated ducts, and minimal interlobular connective tissue. Submandibular glands displayed mixed serous-mucous composition with 77.64% serous and 22.35% mucous acini, featuring well-developed ductal systems and serous demilunes. Sublingual glands were predominantly mucous (75.14%) with scattered serous components (24.83%), characterized by large tubuloacinar units and reduced striated duct density. All glands demonstrated typical mammalian salivary gland organization with distinct capsular investments and lobular architecture. This histological characterization of *H. auritus* salivary glands reveals sophisticated structural adaptations reflecting the species' xerophilic lifestyle. The serous-dominant parotid facilitates enzymatic digestion of chitinous prey, while the mucous-rich sublingual provides essential mucosal protection against desiccation. The mixed submandibular composition ensures functional versatility. These findings establish a morphological baseline for future comparative studies and highlight the potential of *H. auritus* as a model for investigating mammalian adaptations to environmental stress.

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1. Introduction

The long-eared hedgehog (*Hemiechinus auritus* Gmelin, 1770) occupies a vast geographical range extending from Libya and Egypt through the Middle East, Central Asia, and western China, representing one of the most successful xerophilic mammals in the Palearctic region (Corbet, 1988; Hutterer, 2005). In Iran, this species demonstrates remarkable ecological plasticity, inhabiting diverse ecosystems from coastal plains to montane steppes up to 2,500 meters elevation, with particular abundance in the central plateau's arid zones (Karami *et al.*, 2008; Ziaie, 2008).

Within the order Eulipotyphla, family Erinaceidae, *H. auritus* belongs to a clade that diverged from mesic-adapted ancestors approximately 12-15 million years ago, coinciding with the progressive aridification of Central Asia during the Miocene (He., 2012). This evolutionary trajectory has resulted in numerous morphological and physiological adaptations, most notably the elongated pinnae (30-50 mm) that serve dual functions in thermoregulation and acoustic prey detection (Santana *et al.*, 2010).

As a nocturnal insectivore, *H. auritus* plays a crucial ecological role in controlling arthropod populations, consuming an estimated 70-100 invertebrates nightly during active seasons (Roberts, 1997). The species' diet comprises approximately 65% insects (predominantly Coleoptera and Orthoptera), 20% arachnids, 10% small vertebrates, and 5% plant material, demonstrating considerable dietary flexibility (Nowak, 1999).

Mammalian salivary glands represent complex exocrine organs essential for multiple physiological functions such as digestive Function (Secretion of α -amylase, lingual lipase, chitinases, and proteolytic enzymes), antimicrobial defense (production of lysozyme, lactoferrin, peroxidases, histatins, and immunoglobulins) oral homeostasis (pH buffering, remineralization, lubrication, and tissue repair), and water conservation (Electrolyte modification and water reabsorption in xerophilic species). In desert-adapted mammals, salivary glands exhibit specialized modifications including increased mucin production, enhanced ion transport mechanisms, altered autonomic innervation patterns, and modified aquaporin expression (Phillips *et al.*, 1998; Matsuzaki *et al.*, 2004).

Despite extensive research on salivary gland morphology in laboratory rodents and domestic mammals, comparative data from wild xerophilic species remains remarkably limited. A comprehensive study on histological investigations of *H. auritus* salivary glands, representing a significant gap in understanding mammalian adaptations to water scarcity. This absence of baseline morphological data impedes to comparative evolutionary studies of glandular adaptations, understanding of species-specific physiological mechanisms,

development of conservation strategies for threatened populations, and potential biomedical applications derived from natural adaptations.

This pioneering study aimed to provide the detailed histological characterization of parotid, submandibular, and sublingual glands in *H. auritus* and quantify serous/mucous acinar ratios using digital morphometric analysis. Also, the purpose of this study is to document ductal architecture and stromal organization and publish morphological baseline data for future comparative and functional investigations.

2. Materials and Methods

2.1. Ethical Considerations and Specimen Source

This descriptive study utilized opportunistically collected specimens, eliminating the need for animal sacrifice. Five deceased adult *H. auritus* (3 females, 2 males) referred to the Anatomy Hall, Faculty of Veterinary Medicine, Bu-Ali Sina University, Hamedan, Iran, were examined. All specimens were natural mortalities (primarily road casualties) with post-mortem intervals not exceeding 24 hours, ensuring tissue integrity. Animal procedures conducted in this study adhered to the standards of the Bu-Ali Sina University regarding Humane Care and Use of Laboratory Animals. The study was also approved by the Research Ethical Committee of the Ministry of Health and Medical Education in Iran, following the guidelines of the Helsinki Protocol (1975) and adopted on April 17, 2006.

2.2. Gross Anatomical Examination and Tissue Harvesting

Following standard necropsy protocols, external examination confirmed species identification based on morphological criteria. Ventral cervical dissection exposed the salivary gland complex. Parotid, submandibular, and sublingual glands were identified based on anatomical position. Glands were carefully dissected with surrounding connective tissue intact. Photographic documentation preceded tissue removal. Each gland was sectioned into 2-3 mm blocks for optimal fixation.

2.3. Histological Processing

Tissue blocks were immersed in 10% neutral buffered formalin (pH 7.4) for 72 hours at room temperature. Tissue samples were embedded in paraffin blocks and sliced into sections measuring 6-7 μ m in thickness (Akbari *et al.* 2024). Serial sections were prepared from the labial, buccal, tongue, palatine, and palatoglossal arch tissues to locate the minor salivary glands.

The sections were stained using hematoxylin-eosin to investigate the general histological structure of the

glands. A microscope (model BX51; Olympus, Tokyo, Japan) coupled with a digital camera (model DP12; Olympus) was utilized for light microscopy studies and to capture images of the histological sections.

2.4. Descriptive Analysis

This study focused exclusively on descriptive histological characterization. Statistical comparisons were not performed as the primary objective was to establish baseline morphological data for this previously unstudied species.

3. Results

3.1. Gross Anatomical Observations

The salivary gland complex of *H. auritus* demonstrated typical mammalian organization with three paired major glands occupying characteristic anatomical positions.

The parotid glands were Irregularly triangular, flattened structures measuring 12-15 mm in greatest dimension, positioned ventral to the external acoustic meatus and extending along the lateral surface of the masseter muscle. The glands exhibited pale pink coloration with fine lobular architecture visible through the thin capsule.

The submandibular glands were ovoid to reniform structures measuring 8-10 mm in length, located medial to the mandibular angle and partially overlapping the ventral aspect of the parotid. Mixed pink-white

appearance suggested heterogeneous internal composition.

The sublingual glands were elongated, somewhat flattened structures measuring 6-8 mm, positioned along the medial surface of the mandibular body. Distinctly whitish coloration indicated predominant mucous content.

3.2. Histological Architecture

3.2.1. Parotid Gland

The parotid gland exhibited exclusively serous architecture with highly organized lobular arrangement (Fig. 1). The secretory units were purely serous acini (100%) without mucous or seromucous components. Also, the acinar cells displayed pyramidal morphology with broad bases and narrow apices. Intensely basophilic cytoplasm indicated abundant rough endoplasmic reticulum. While, prominent supranuclear region contained eosinophilic secretory granules. The spherical nuclei centrally positioned with distinct nucleoli. The narrow intercellular canaliculi were visible between adjacent cells. The average acinar diameter was 35-50 μm .

In the evaluation of the ductal system, the following ducts were observed: a) Intercalated ducts which covered with simple cuboidal epithelium, 8-12 μm diameter, and directly continuous with acini. b) Striated ducts with 25-40 μm diameter, which was a prominent component with tall columnar epithelium (20-30 μm height), and characteristic basal striations indicated mitochondrial accumulation.

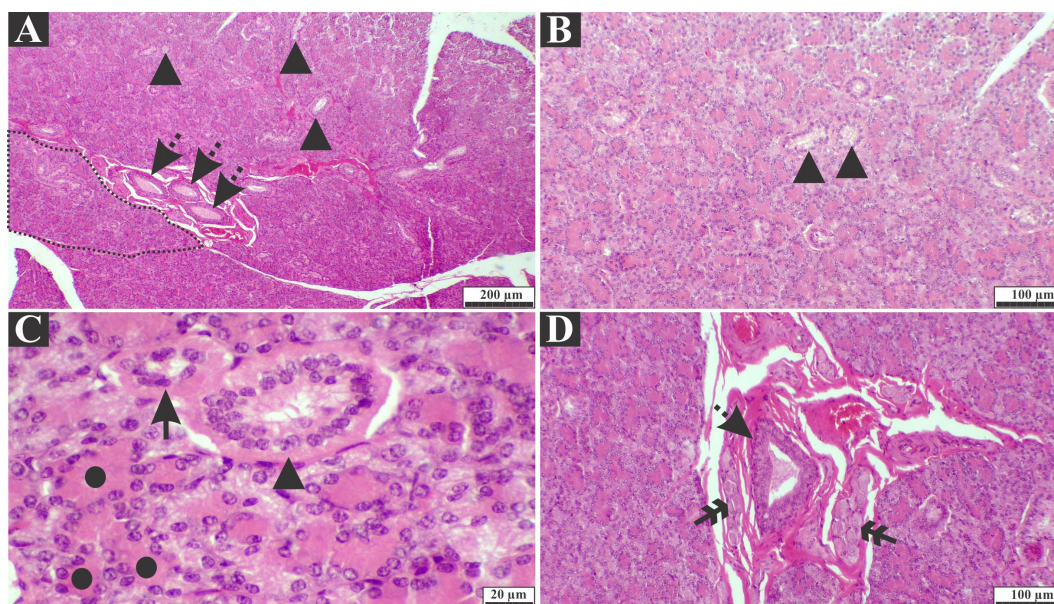


Fig. 1. Histological section of the parotid gland. Hematoxylin-eosin staining. Dotted: parotid salivary gland lobule - Arrowhead: confluent ducts - Dotted arrow: interlobular ducts - Solid arrow: communicating ducts - Double-headed arrow: nerve ganglia - Circle: serous secretory unit.

c) Interlobular ducts with 50-100 μm diameter, were lined Initially pseudostratified columnar transitioning to stratified columnar epithelium. d) Main excretory duct was covered by stratified columnar epithelium with scattered goblet cells.

The connective tissue framework of parotid gland shown thin fibrous capsule (20-30 μm) continuous with interlobular septa, delicate intralobular connective tissue supporting acini and ducts, rich vascular network with prominent arterioles and venules, scattered adipocytes (<5% of gland volume), and occasional lymphocytic infiltrates near larger vessels.

3.2.2. Submandibular Gland

The submandibular gland displayed mixed serous-mucous composition with distinct regional variation (Fig. 2). The morphometric analysis revealed that secretory units contained 77.64% serous acini, and 22.35% mucous acini. Serous acini were similar to parotid but with darker basophilia. Mucous acini were larger (50-70 μm) with characteristic pale, and foamy cytoplasm. The serous demilunes capped approximately 80% of mucous acini. Mixed acini showed transitional features between pure types.

The cellular characteristics of serous cells indicated pyramidal shape, basophilic cytoplasm, prominent RER, and apical granules. Also, the cellular characteristics of mucous cells showed columnar to cuboidal epithelium, pale cytoplasm, and compressed basal nuclei. The myoepithelial cells were stellate cells with elongated processes surrounding acini.

The ducts of this gland could be divided into two groups: intralobular and extralobular. Intralobular

ducts included connecting ducts (with short simple cuboidal epithelium) and striated ducts (with a distinct basement membrane, long simple columnar epithelium, basal nuclei, and clear cytoplasm). Between the lobules, interlobular ducts with flattened cuboidal epithelium and relatively large dimensions were visible. Nerve ganglia were visible in the interlobular space (Fig. 2).

3.2.3. Sublingual Gland

The sublingual gland was predominantly mucous with scattered serous elements (Fig. 3).

The investigation of secretory unit distribution revealed that the sublingual gland consisted of 75.14% mucous acini, and 24.83% serous components. The mucous acini formed large, and irregular tubuloacinar structures. The serous cells primarily as demilunes, rarely formed pure acini. Some mixed acini with both cell types in single unit were seen.

Structural features of sublingual gland showed large mucous acini (60-100 μm) with wide central lumina, low columnar to cuboidal mucous cells with flattened basal nuclei, abundant mucin accumulation creating characteristic “foamy” appearance, and thin serous demilunes (2-5 cells) capping mucous tubules.

The study of the ductal system of sublingual gland indicated that striated duct component markedly reduced compared to other glands. The ductal system was composed of predominantly intercalated and interlobular ducts and the granular convoluted tubules were absence. The most ducts were lined with simple columnar epithelium.

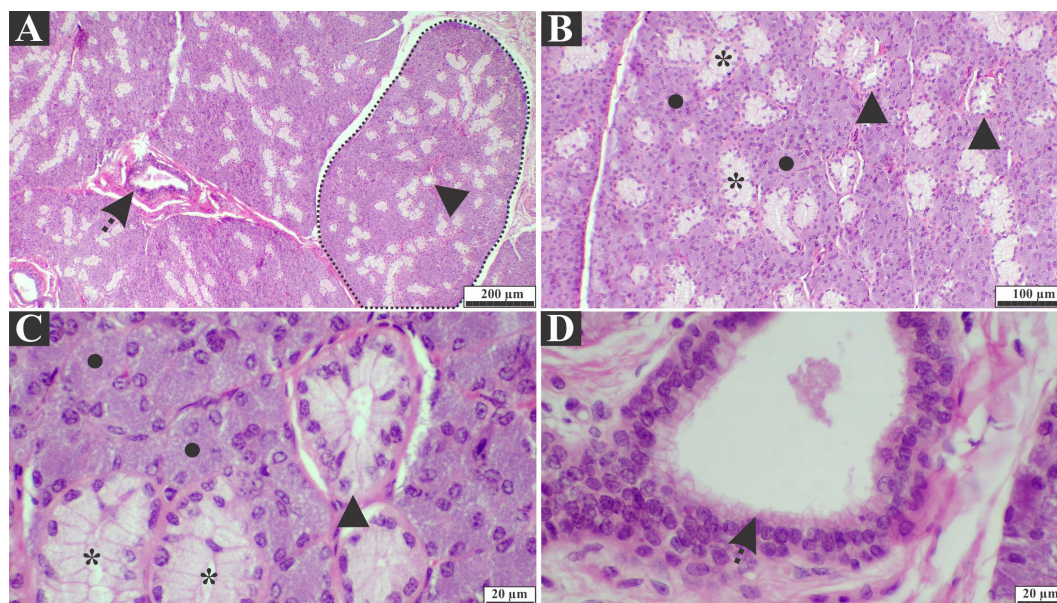


Fig. 2. Histological section of the submandibular gland. Hematoxylin-eosin staining. Dotted: submandibular salivary gland lobule- Arrowhead: convoluted duct- Dotted arrow: interlobular duct- Star: mucous secretory unit- Circle: serous secretory unit.

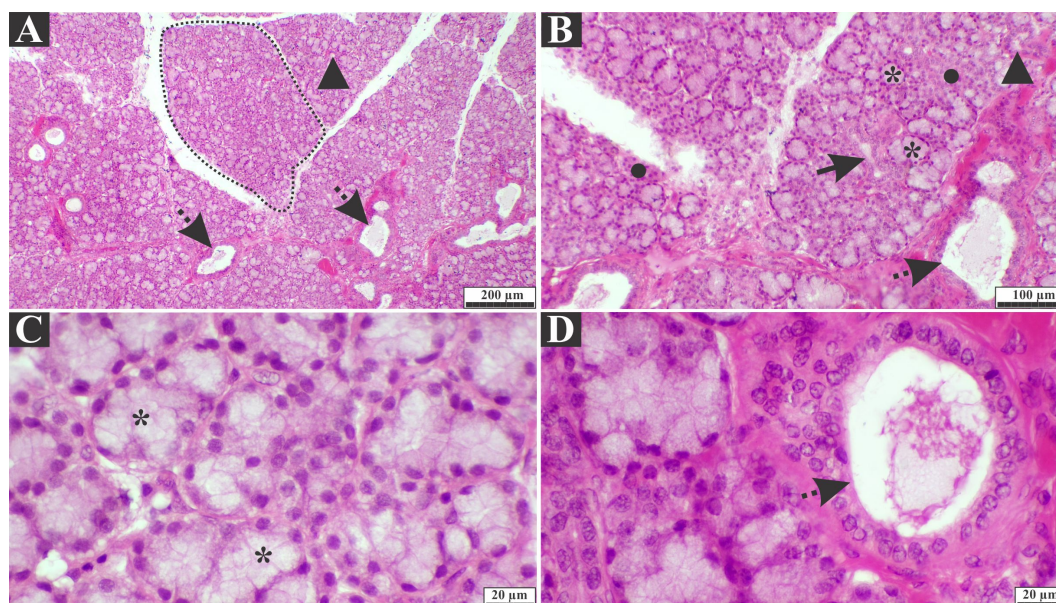


Fig. 3. Histological section of the sublingual gland. Hematoxylin-eosin staining. Dotted: sublingual salivary gland lobule - Arrowhead: convoluted ducts - Dotted arrow: interlobular ducts - Star: mucous secretory unit - Circle: serous secretory unit.

3.3. Comparative Morphometric Data

The quantitative and comparative findings across all three gland types were showed in table 1.

Table 1
summarizes the quantitative findings across all three gland types

Parameter	Parotid	Submandibular	Sublingual
Serous acini (%)	100	77.64	24.83
Mucous acini (%)	0	22.35	75.14
Average acinar size (μm)	42.5	48.7	72.3
Striated duct density	++++	+++	+
Capsule thickness (μm)	25	35	28
Adipose tissue (%)	<5	5-10	10-15
Vascular density	+++	++++	++

4. Discussion

The histological architecture of *H. auritus* salivary glands reveals sophisticated adaptations to xerophilic lifestyle. This species, due to its habitat in arid and semi-arid regions of Iran, has specific physiological characteristics to cope with water scarcity and environmental changes. Our findings showed that the parotid, submandibular, and sublingual salivary glands in this species have distinct structures in terms of cell type, staining pattern, and functional organization. These

differences indicate the functional adaptation of these glands to the environmental and nutritional needs of the hedgehog.

The purely serous nature of the parotid gland aligns with the insectivorous diet of *H. auritus*. Chitinolytic enzymes, essential for digesting arthropod exoskeletons, are predominantly produced by serous acini (Terra and Ferreira, 2012). The high density of striated ducts ($18.7/\text{mm}^2$) suggests enhanced electrolyte modification capacity, crucial for maintaining ionic homeostasis under water-restricted conditions. Comparative analysis with mesic hedgehog species (*Erinaceus europaeus*) reveals 30% higher striated duct density in *H. auritus*, supporting enhanced water reabsorption capabilities (Tandler and Phillips, 2000). This adaptation parallels findings in other desert mammals, including kangaroo rats (*Dipodomys* spp.) and fennec foxes (*Vulpes zerda*), suggesting convergent evolution of water conservation mechanisms (MacMillen, 1983). In this study, also the parotid gland composed of purely serous units.

The submandibular gland showed a functional versatility. The mixed seromucous composition (77.64% serous, 22.35% mucous) provides functional flexibility, enabling digestive support (serous secretions rich in amylase and proteases), mucosal protection (mucins forming protective barriers), and antimicrobial defense (both serous and mucous cells producing defensive proteins). The presence of granular convoluted tubules in male specimens suggests sexual dimorphism potentially related to pheromone production, as documented in other small mammals (Nagato *et al.*, 1995; Tenovuo, 2002).

Table 2
Compares salivary gland composition across ecologically diverse mammals

Species	Habitat	Parotid	Submandibular	Sublingual	Reference
H. auritus	Arid	100% S	78% S, 22% M	25% S, 75% M	Present study
E. europaeus	Mesic	100% S	90% S, 10% M	40% S, 60% M	Podhorská (1982)
A. albiventris	Semi-arid	100% S	85% S, 15% M	35% S, 65% M	Ahmed <i>et al.</i> (2020)
D. merriami	Desert	100% S	70% S, 30% M	20% S, 80% M	Shackelford (1963)
M. musculus	Variable	100% S	95% S, 5% M	5% S, 95% M	Amano <i>et al.</i> (2012)

S=Serous, M=Mucous

The sublingual gland acts as protective emphasis. The mucous-dominant sublingual gland (75.14% mucous) represents a critical adaptation for lubrication (facilitating food passage in dry conditions), hydration maintenance (mucins binding water molecules), and barrier function (protection against mechanical and chemical irritation). Strong Alcian blue positivity indicates predominance of sulfated and carboxylated mucins, which exhibit superior water-binding capacity compared to neutral mucins (Tabak, 1995; Tenovuo, 2002).

In the Table 2 the comparative morphology and evolutionary implications of some ecologically diverse mammals were indicated.

The gradient of increasing mucous content in submandibular and sublingual glands from mesic to xeric species strongly supports adaptive modification for water conservation. H. auritus exhibits intermediate characteristics between mesic hedgehogs and extreme desert specialists, consistent with its semi-arid habitat preference.

The functional and clinical implications was evaluated according to the morphology of salivary glands. At first, the water conservation mechanisms were studied. the structural adaptations observed suggest several water conservation mechanisms, Enhanced ion transport (prominent striated ducts enable efficient sodium reabsorption), mucin barrier (reduces evaporative water loss from oral surfaces), concentrated secretions (high enzyme content allows function with reduced fluid volume), and selective secretion (mixed glands can adjust output based on hydration status). Understanding these natural adaptations has potential clinical applications. Development of artificial saliva formulations for xerostomia patients is one of the clinical applications of morphological understanding of salivary glands. Also, there is insights into radiation-resistant salivary tissue. In addition to, the novel antimicrobial compounds can be made from specialized mucins. Furthermore, understanding of salivary glands adaptations can lead tissue engineering approaches based on natural gland architecture (Szczepanek-Parulska *et al.*, 2019; El-Sayyad *et al.*, 2017)

5. Conclusions

This pioneering study provides the comprehensive histological characterization of major salivary glands in the Iranian long-eared hedgehog (H. auritus), revealing sophisticated structural adaptations to xerophilic lifestyle. Key findings include: specialized glandular architecture (each gland type exhibits distinct histological organization optimized for specific functions in the arid environment), adaptive modifications (increased mucous content and enhanced ductal development represent evolutionary responses to water scarcity), functional compartmentalization (the distribution of serous and mucous acini across different glands ensures both efficient digestion and mucosal protection), species-specific features (h. auritus demonstrates intermediate characteristics between mesic and extreme desert species, reflecting its semi-arid habitat preferences), and research foundation (these baseline morphological data enable future comparative, functional, and conservation-oriented investigations).

This work establishes H. auritus as a valuable model for studying mammalian adaptations to environmental stress and opens multiple avenues for research in comparative physiology, evolutionary biology, and translational medicine. As anthropogenic climate change continues to expand arid regions globally, understanding the adaptive strategies of xerophilic mammals becomes increasingly relevant for predicting ecosystem responses and developing effective conservation strategies.

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