Original Article

Immunohistochemical Assessment of GDNF and Chromogranin A Expression in Erosive and Granulomatous Lesions in Glandular Region of Equine Stomach

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ABSTRACT

The equine stomach consists of two separate non-glandular and glandular sections. Despite the incidence of most lesions in the non-glandular region, both stomach parts are prone to lesions. In this study, 41 hybrid-native horses, including 24 stallions and 17 mares, were examined over five years. In total, 27 horses (65.85%) that were sampled had lesions, including erosion, granuloma, or both on the glandular region of the stomach. Occurrence of gastric erosive and granulomatous lesions had no significant relationship with the age and gender of horses or the sampling season (P>0.05). Moreover, parasites *Gastrophilus* and *Habronema* were mainly the primary cause of gastric erosive and granulomatous lesions respectively. In Periodic Acid Schiff (PAS) stained tissue sections, the inflammation severity in granulomatous lesions was higher and statistically significant, compared to erosive lesions (P<0.05). Immunohistochemistry revealed negative expression of glial cell line-derived neurotrophic factor in gastric lesions, while its expression was relatively positive in normal stomachs. Interestingly, based on counting cells and evaluation of expression intensity, Chromogranin A expression in neuroendocrine glandular cells had a significant relationship with the increase of severity and depth of the lesions (P<0.05). The results indicated that the glial cell line-derived neurotrophic factor does not affect the pathogenesis of equine gastric lesions while confirming the role of increment of gastric neuroendocrine cells in lesion progress. Furthermore, the increased expression of Ki67 and p53 proteins in granulomatous lesions, compared to other groups, may be associated with the proliferation and control process of the cells in measures regarding the formation and healing of the lesion.

Keywords: Chromogranin A, Erosion, Granulomatous lesion, Glial cell line-derived neurotrophic factor, Horse stomach

1. Introduction

Equine gastric lesions occur in both the glandular and squamous sections. The pathophysiology of gastric ulcers is related to a lack of balance between the progressive (acid and pepsin) and defensive factors (1). The neuroendocrine cells are scattered among the epithelial cells of the gastrointestinal system, which play a vital role along with the enteric nervous system in the function of the digestive system. Multiple immunohistochemical studies have been conducted on the distribution and relative frequency of neurons and neuroendocrine cells, including neurotransmitters and neuropeptides, in the digestive system of various mammals, namely humans, horses, pigs, guinea pigs, buffaloes, dogs, camels, mice, deer, and rats, to determine their performance (2).

The glial cell line-derived neurotrophic factor (GDNF) and shared GFRα receptors play roles in the growth of the enteric nervous system which activates a receptor complex comprised of RET and GFRα shared receptor and initiates exclusive signal transduction (3). Chromogranin A (CgA) belongs to the chromogranin/secretogranin acidic secretory proteins extensively found in the diffuse endocrine system of the gastrointestinal system, respiratory system, endocrine glands (adrenal and pituitary gland), and groups of endocrine cells in glandular tissues of pancreas and thyroid. Indeed, CgA secretion depends on the type of the cell and the number of present secretory granules inside the cell (4).

Various studies have suggested alterations in p53 expression in pre-cancerous gastric lesions (such as atrophy or metaplasia) as efforts to stop the cycle of the damaged cell, and its build-up (lack of expression) indicates its dysfunction as a cell proliferation suppressor. Increased p53 expression may be a part of a protective mechanism against the host, arresting the cell cycle to repair the cell genome (5). The Ki67 is a nucleus antigen expressed in every cell cycle phase (except for the G0 phase). Moreover, it should be noted that Ki67 is a marker of proliferating cells. In immunohistochemistry, to measure the proliferation ratio of normal, inflammatory, and tumor tissues, a monoclonal antibody (MIB-1) is extensively used against the Ki67 (6).

The current study aimed to investigate the frequency of gastric erosion and ulcers of native-hybrid horses from rural areas of Urmia city in Iran that were referred to the veterinary hospital of Islamic Azad University, Urmia Branch, Iran over five years. The investigation was based on the histopathology results of paraffin blocks of tissue samples obtained from necropsy or post-operative samples. Furthermore, the pathological changes of gastric wall glands and their tissue in erosions and granulomatous lesions were comparatively analyzed using the Periodic acid–Schiff (PAS) method.

In addition, the immune reaction of gland cells and gastric tissue in tissue samples with erosions and granulomatous lesions were evaluated and compared with GDNF, CgA, S100, P53, and Ki67 antibodies. This study also aimed to investigate the role of nerve growth factors in equine gastric erosions and ulcer pathogenesis, the function of neuroendocrine cells (argentaffin cells) in lesion formation, dendritic cell status, intracellular alterations, and cell proliferation rate.

2. Materials and Methods

2.1. Design and Sampling

The sampling was conducted over five years (June 2017 to June 2022) on 41 native-hybrid horses (mostly hybrid with Kurdish breed) from rural areas of Urmia city at a specialized veterinary hospital. The samples were obtained using biopsy (following the abdominal surgery in the Surgery Department) and necropsy during the mentioned period and stored in the Pathology Department. For horse euthanasia, a barbiturate overdose was administered intravenously (7, 8) and the necropsy was conducted using the standard suggested protocol.

Besides, to investigate hematological indicators (including cell blood count), blood samples were obtained from the jugular vein. After preparation of a

blood smear, fixation, and Giemsa staining, the slides were investigated with a 100x objective lens to calculate a white blood cell differential, and the percentage of each white blood cell was registered. The tissues were placed in a 10% formalin buffer solution (with a 1:10 tissue-to-fixative V/V ratio) for tissue fixation.

2.2. Pathology

The tissue sections were sent to the pathology laboratory for preparations and staining. Paraffin blocks were prepared 48 h after the tissue fixation and passage (dehydration, clarification, and impregnation). Afterward, sections were made (with 6 μm thickness) using a rotary microtome. Finally, the sections were stained using the PAS method and investigated with light microscopy.

2.3. Immunohistochemistry

In this study, the following antibodies were used for immunohistochemical staining: GDNF (Santa Cruz Biologicals, Dallas, Texas, USA; 1:150), CgA (Dako, Glostrup, Denmark; 1:100), S100 (Dako, Glostrup, Denmark; 1:800), p53 (Elabsciences, USA, 1:300), and Ki67 (Dako, Glostrup, Denmark; 1:150). Briefly, 4–5-micron sections of the paraffin blocks were prepared, and after being deparaffinized in xylene, the hydration was performed using alcohol and distilled water. Before the staining, heat-induced epitope retrieval was performed. Finally, the immunohistochemical staining was performed following the protocol provided by the manufacturer using EnVision/ horseradish peroxidase method (9).

The suitable tissues were used as positive controls for each antibody and also replaced the primary antibodies with non-immune IgG that were used as the negative controls. The scoring of gastric lesions of the immunohistochemical sections was based on the study conducted by Rodrigues, Dore (10). Accordingly, based on the equine gastric cells with erosion and granulomatous lesions that were positive for immunohistochemical markers, they were scored based on a 0-3 scale, in which the scores 0, 1, 2, and 3 indicated lack of lesion, 1-10% positive cells, 11-30% positive cells, and more than 31% positive cells, respectively (10). The inflammation severity scoring system was as follows: a) 0-3 (inflammatory cell) = absent (-) (normal), b) 4-8 (inflammatory cell) = mild (+), c) 9-24 (inflammatory cell) = moderate (++), and d) >25 (inflammatory cell) = severe (+++) (10).

2.4. Statistical Analysis

The statistical analysis was performed in IBM SPSS (version 26). The descriptive findings of the studied variables were calculated and reported, including indices, such as absolute frequency, relative frequency, mean, standard error, and standard deviation. For quantitative data, the normality of the data was assessed using the Kolmogorov-Smirnov test. Afterward, the homogeneity of variance was evaluated using Levene's test. Given the normal distribution of the data of blood factors of the study groups, the difference between the mean blood factors of the studied groups was investigated using the one-way analysis of variance (ANOVA) and Tukey post hoc test.

In addition, Kruskal-Wallis statistical test was used to compare the qualitative variables of histopathology and histochemistry of the treatments. The Mann-Whitney U test was used for pair-wise comparison of the dependent variables. The qualitative data were summarized using the median (25^{th} percentile and 75^{th} percentile). The difference between the studied groups in terms of average scores of chromogranin staining was investigated using one-way ANOVA and Tukey post hoc test. Moreover, chi-square and Fisher's exact tests were used to investigate the association of the demographic and pathologic indices of the studied horses with the frequency of lesions.

3. Results

3.1. Prevalence and Cases of the Gastric Lesions

In total, 41 native-hybrid horses were sampled by biopsy or necropsy during the five years of the study (June 2017 to June 2022), 24 and 17 of which were stallions and mares, respectively. Among the sample group, 27 horses

(65.85), including 15 stallions (55.6%) and 12 mares (44.4%), indicated erosion, ulcer, or both on the glandular region of the stomach. In this study, 27 horses had gastric lesions, 8 (29.6%) and 19 (70.4%) of which were below and above eight years old, respectively.

Moreover, necropsy revealed that the relative frequency of the lesions (erosion or ulcer) was the highest in gastric cardia (29.6%), fundus (14.8%), antrum, and pylorus (11.1%). However, there was no significant relationship between the type and location of the lesion (P>0.05). According to the findings, the relative frequencies of horses with gastric lesions ranked by season were 55.6%, 33.3%, 7.4%, and 3.7% in spring, autumn, summer, and winter, respectively. However, there was no significant relationship between the existence of gastric lesions and the sampling season (P>0.05).

Given the cause of the gastric lesion, the following factors were the most frequent in the mentioned order: *Gasterophilus* parasite (n=8, 29.6%), *Habronema* parasite (n=6, 22.2%), unknown (n=5, 18.5%), phytobezoar (n=3, 11.1%), concurrent *Gasterophilus* and *Habronema* parasites (n=2, 7.4%), long-term administration of non-steroidal anti-inflammatory drugs (NSAIDs) (n=2, 7.4%), and stress and excessive physical activity (n=1, 3.7%). It must be noted that lesions caused by *Gasterophilus* and *Habronema* parasites were erosion and granulomatous, respectively. In other words, a notable portion of the equine gastric erosions and granulomatous lesions were caused by parasitic gastritis.

3.2. Hematology

Table 1 summarizes the results of comparing the blood indices of horses with gastric lesions and those of horses with normal stomachs. Accordingly, except for the mean corpuscular hemoglobin concentration (MCHC), lymphocyte (LYM), and basophil indices, the other indices indicated a significant difference between the various groups (P<0.05).

3.3. Histopathology and Immunohistochemistry

Figure 1 illustrates the results of the changes in the severity of inflammation relating to erosion and ulcers in the glandular region of the studied horses. Figure 2 depicts the gross appearance of some erosive and granulomatous gastric lesions in the necropsied equine. The comparative histochemical results related to normal and lesioned stomachs are shown in figure 3. Table 2 tabulates the results of the comparison of the horses with gastric lesions and those with normal stomachs in terms of the expression of their immunohistochemical markers. Moreover, the results of immunolabelling by GDNF, Cg A, and S100 antibodies are exhibited in figure 4. Besides, immunohistochemical expressions of P53 and Ki67 in the normal and lesioned stomach tissues are presented in figure 5.

Table 1. Comparing the mean ± standard deviation of blood indices relating the gastric erosions and ulcers of the glandular region of the studied horses

Parameter	Sampled horse				Significance level (P- value)
	Normal	Erosion	Granulomatous	Erosion+Granulomatous	
HCT (%)	33.07 ± 1.52^{a}	33.62 ± 0.92^{a}	29.14 ± 0.98^{b}	28.50 ± 0.64^{b}	0.044
Hb (g/l)	134.21 ± 2.33^{c}	124.18 ± 2.10^{b}	113.14 ± 2.89^{a}	111.00 ± 2.79^{a}	0.001
RBC (10^{12}/l)	7.75 ± 0.28^{b}	7.40 ± 0.14^{ab}	6.69 ± 0.14^{a}	6.66 ± 0.20^{a}	0.012
MCV (fl)	43.96 ± 0.92^{a}	45.28 ± 0.85^{a}	49.39 ± 0.97^{b}	50.45 ± 0.63^{b}	0.001
MCHC (g/l)	336.78 ± 10.08	347.62 ± 8.66	349.57 ± 16.03	375.75 ± 11.40	0.318
PLT (10^{9}/l)	261.50 ± 16.28^{a}	330.56 ± 16.39^{ab}	361.85 ± 18.42^{b}	349.50 ± 5.33^{b}	0.001
WBC (10^{9}/l)	9.25 ± 0.4^{a}	10.23 ± 0.47^{a}	11.36 ± 1.06^{ab}	13.13 ± 1.09^{b}	0.007
Neu (10^{9}/l)	4.09 ± 0.22^{a}	5.15 ± 0.21^{a}	7.70 ± 0.40^{b}	8.03 ± 0.44^{b}	0.001
Lym (10^{9}/l)	3.03 ± 0.17	2.99 ± 0.14	2.97 ± 0.33	2.76 ± 0.41	0.916
Mon (10^{9}/l)	0.23 ± 0.03^{a}	0.43 ± 0.04^{b}	0.47 ± 0.06^{b}	0.44 ± 0.08^{b}	0.001
Eos (10^{9}/l)	0.23 ± 0.01^{a}	0.54 ± 0.30^{b}	0.73 ± 0.05^{c}	0.70 ± 0.06^{c}	0.001
Bas (10^{9}/l)	0.035 ± 0.013	0.025 ± 0.011	0.057 ± 0.029	0.025 ± 0.025	0.604

Note: P- values less than 0.05 (P<0.05) were considered significant. Different letters in each column indicate a significant difference between the groups

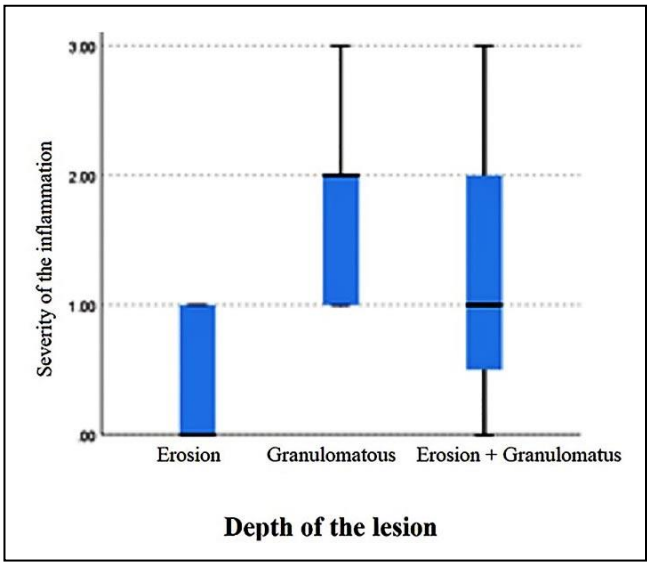

Figure 1. The diagram of the severity of inflammation is based on the gastric erosions and ulcers of the glandular region of the studied horses. The figure indicates that most gastric erosions had inflammation severity of 0 to +, granulomatous lesions had inflammation severity of + to ++, and the gastric erosions concurrent with granulomatous lesions had inflammation severity of + to ++. Also, the pair-wise comparison using the Mann-Whitney test indicated that the inflammation between the group with erosion and the group with the granulomatous lesion (P=0.001) had a significant relationship (P<0.01). However, there was no statistically significant relationship between the group with erosion and the group with erosion+granulomatous (P=0.086). Also, there was no significant difference in the severity of inflammation between the group with granulomatous lesions and the group with erosion+granulomatous (P=0.322)

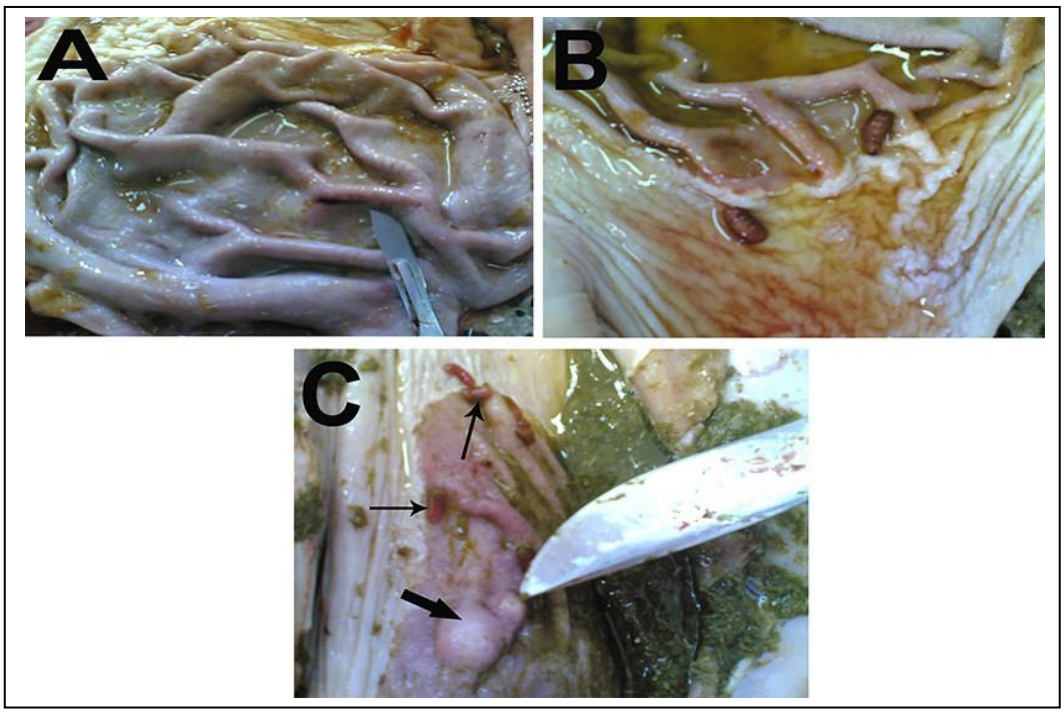

Figure 2. The appearance of several equine gastric erosions and granulomatous lesions during the necropsy. **A)** Presence of erosion in the glandular region of the stomach. **B)** Presence of erosion at *Gastrophilus* parasite attachment site. **C)** Concurrent presence of *Gastrophilus*-caused erosions (the small arrows) and granulomatous lesions (the large arrow) caused by the *Habronema muscae*.

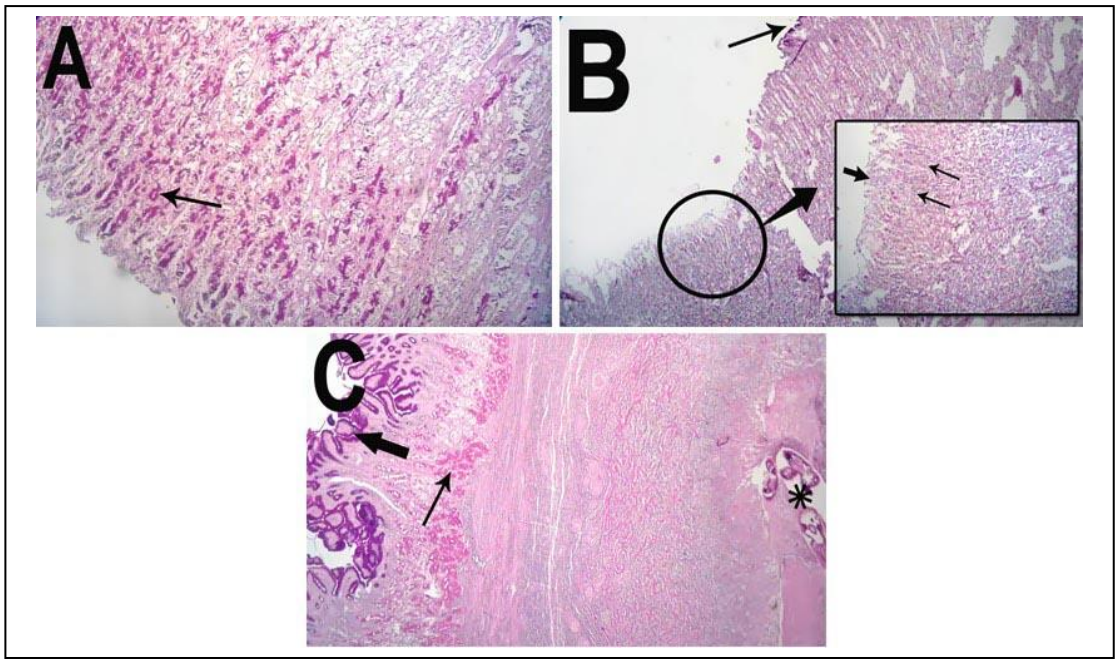

Figure 3. Comparison of histochemical changes in tissue sections of equine gastric erosions and granulomatous lesions. **A)** Normal stomach: Gastric glands with regular structure, positive PAS staining (arrow), and normal surface glands. **B)** Stomach with erosion; primary picture: Surface glands with healthy columnar epithelial cells (arrow) close to the lesion and the circled area indicating gastric erosion with visible necrotic cells. Internal picture: larger view of the circled area on the primary picture, showing necrotic epithelial cells, cell debris (thick arrow), and a few inflammatory cells (thin arrow) among the gastric glands. **C)** Stomach with deep granulomatous lesion: Healthy gastric glands on the surface with positive PAS (large arrow). Due to the formation of granuloma and its pressure on the higher layers (closer to the surface), the lower glands have decreased (atrophy), and their tonality compared with PAS is lower than the normal stomach. The picture shows thick layers of connective tissues (collagen), muscles, and a large number of inflammatory cells inside the gland sublayer. The picture also shows sections of the *Habronema* parasite (asterisk) causing granuloma formation. (A: ×10 zoom, B and C: ×4 zoom (internal picture with ×40 zoom); PAS staining)

Table 2. Comparison of the immunohistochemical results relating the gastric erosions and ulcers of the glandular region of the studied horses using the Kruskal-Wallis test

Lesion type	Antibody			
	GDNF	P53	Ki67	S100
Normal (n=10)	2.0 (1.75, 2.0) [b]	0.5 (0.0, 1.0) [a]	0.0 (0.0, 0.25) [a]	0.0 (0.0, 1.0) [a]
Erosion (n=10)	0.0 (0.0, 0.0) [a]	1.0 (0.0, 0.0) [a]	2.0 (1.75, 2.0) [b]	0.0 (0.0, 1.0) [a]
Granulomatous (n=10)	0.0 (0.0, 0.0) [a]	2.0 (1.75, 2.0) [b]	1.0 (1.0, 2.0) [b]	1.0 (1.0, 1.25) [b]
P- value	0.001	0.001	0.001	0.001

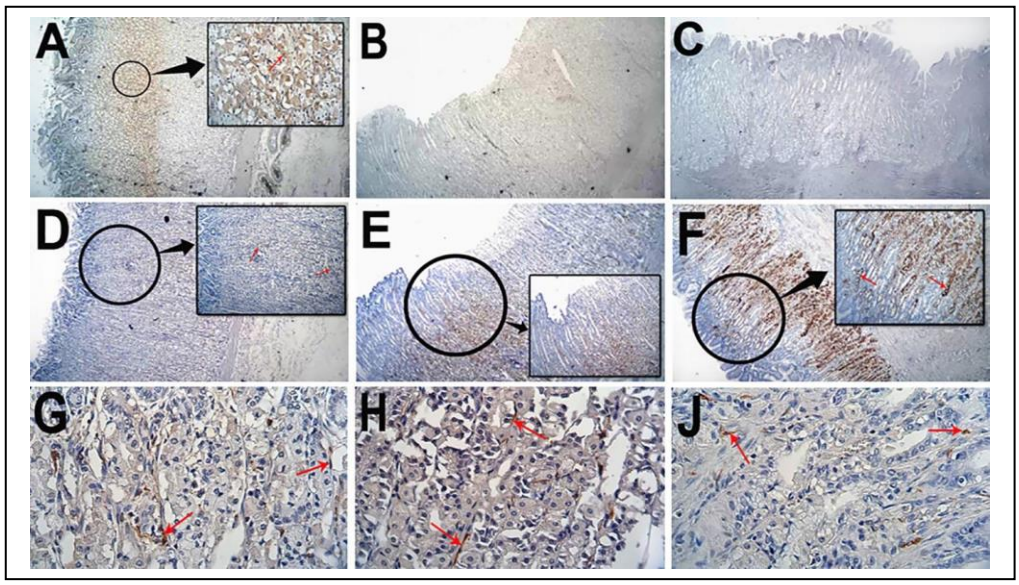

Figure 4. Immunolabeling of tissue sections of equine stomachs with erosions and granulomatous lesions. **A)** Normal stomach: Moderate immune reaction to GDNF in cells of lower and middle gastric glands with visible brown tonality (arrow); the internal picture shows the circled area of the primary picture with higher zoom. **B)** Stomach with erosion: cells with negative immunolabeling response to GDNF. **C)** Stomach with granulomatous lesions: Negative immune reaction of gland cells to GDNF. **D)** Normal stomach: Mild immunolabeling of gland cells at the middle or lower sections of the stomach. The internal picture shows CgA-positive (with low intensity) neuroendocrine cells (arrow) with a higher zoom. **E)** Stomach with erosion: Gastric glandular neuroendocrine cells with positive reaction (with high intensity) to Chromogranin A in the middle and lower sections close to the healthy edges of the lesion. The internal picture showing the circled area with a higher zoom reveals lower intensity immune reaction and fewer cells in the glands of the lesion zone. **F)** Stomach with granulomatous lesion: The picture shows a severe immune reaction to chromogranin A (red arrow) in gastric glandular neuroendocrine cells of every layer (lower, middle, and upper layer). **G)** Normal stomach: Immunolabeling of a few cells around the stomach glands (red arrow) (dendritic cells with S100). **H)** Stomach with erosion: Positive reaction of a few dendritic cells around the stomach glands (red arrow) to S100. **J)** Stomach with granulomatous lesion: Immune reaction of several dendritic cells around the stomach glands (red arrow) to S100. (A, B, C: GDNF staining, ×4 zoom and internal picture with ×40 magnification; DEF: Chromogranin A staining, ×4 magnification and internal picture with ×10 magnification; GHJ: S100 staining, ×40 magnification; IHC)

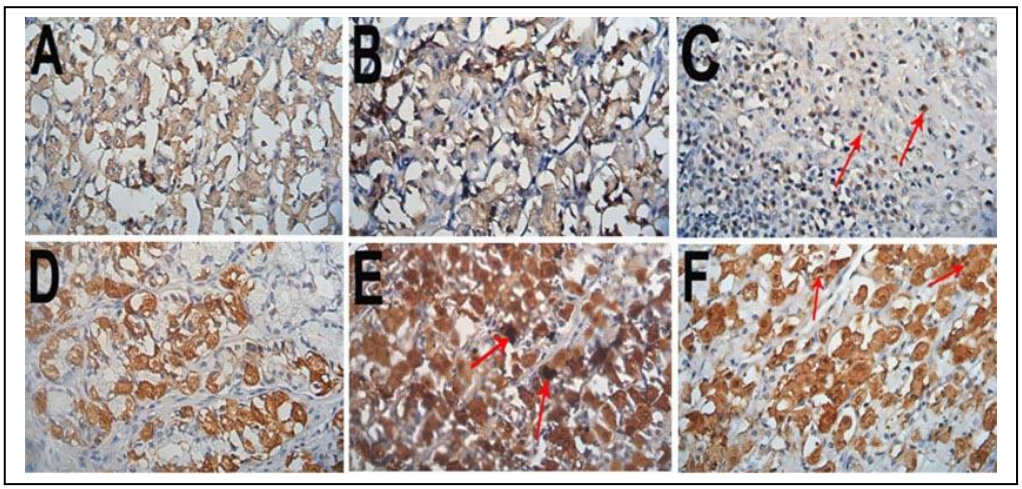

Figure 5. Comparative immunohistochemical staining of equine stomachs with erosion and granulomatous lesion. **A)** Normal stomach: Gland cells with negative reaction to p53. **B)** Stomach with erosion: Gland cells with negative reaction to p53. **C)** Stomach with granulomatous lesions: Fibroblast cells with a positive immune reaction to p53 (red arrow) inside the granuloma tissue. **D)** Normal stomach: Negative nucleus immune reaction of gland cells to Ki67 proliferation marker. **E)** Stomach with erosion: Positive nucleus immune reaction of gastric gland cells to Ki67 proliferation marker. **F)** Stomach with granulomatous lesion: Positive nucleus immune reaction of gastric gland cells to Ki67 proliferation marker. A, B, C: P53 staining, ×40, D, E, F: Ki67 staining, ×40; IHC

The pairwise comparison using the Mann-Whitney method indicated that the difference among the GDNF immunohistochemical factor of the normal group, that of the group with erosion (P=0.001), and that of the group with the granulomatous lesion (P=0.001) was statistically significant (P<0.01). However, there was no significant difference between the group with erosion and the group with the granulomatous lesion (P=0.743) regarding the GDNF immunohistochemical factor value (Table 2).

In the stained sections with immunohistochemical methods, three random microscopic fields (without overlap) were investigated with x40 optical zoom inside an area of 1×1 mm^2 to count the number of CgA-positive cells of each tissue sample (Table 3). The comparison results of CgA staining of healthy horses and horses with lesions (erosions and granulomatous lesions) using the ANOVA test indicated a significant difference between the studied groups (P<0.01). Moreover, Tukey's post hoc analysis revealed that the average CgA staining score was significantly lower in normal samples, compared to that in groups with gastric erosions and granulomatous lesions. Besides, the average CgA staining score of the group with gastric erosion was significantly lower than that of samples with granulomatous lesions (P<0.05) (Table 3).

Table 3. Comparison of mean ± standard deviation of chromogranin A staining results relating the gastric erosions and ulcers of the glandular region of the studied horses

Lesion type	Chromogrnin A (Number of immunopositive cells in 1×1 mm^2 of tissue)
Normal	2.69 ± 0.57 [a]
Erosion	23.44 ±1.15 [b]
Granulomatous	49.38 ±2.62 [c]
P- value	0.001

There were significant differences between groups with different codes (superscript letters [a, b, c]; P < 0.05)

4. Discussion

The results of the present study performed over five years on 27 stomachs of native-hybrid horses indicated gastric erosion and granulomatous lesions. The primary cause of the lesions (erosions and granulomatous lesions) was parasitic gastritis, including *Gasterophilus nasalis*, *Gasrtophilus intestinalis*, and *Habronema muscae*. Phytobezoars and prolonged administration of NSAIDs were also other notable causes of equine gastric lesions.

The gender and age of the horses had no significant relationship with the frequency of gastric lesions (P>0.05). The results also indicated that gastric lesions were more frequent in spring; however, there was no significant relationship between the season of necropsy/sampling and the frequency of gastric lesions (P>0.05). Furthermore, there was a significant relationship between the frequency of all lesions (erosions, granulomatous, and erosion+granulomatous) and the cause of lesions (P<0.05).

Some lesions were simultaneously present in both parts; however, despite the highest frequency of lesions in the cardiac section of the glandular region, the incidence of granulomatous lesions and erosion + granulomatous lesions had no significant relationship with the anatomic location of the lesions (P>0.05). A study on endoscopic findings of stomach ulcers in rural horses in Tabriz, Iran and their relationship with the *Gasterophilus* parasite revealed that the frequency of ulcers in the non-glandular region of the equine stomach had no relationship with *Gasterophilus*. In other words, due to the low number of *Gasterophilus*-cased gastritis (n=4, 20%) in endoscopic observations, this parasite was not considered one of the primary causes of equine gastric lesions in the non-glandular region (11).

Unlike the results of the above-mentioned study, those of the present research suggested *Gasterophilus* as one of the primary causes of parasitic gastritis in the glandular stomach and equine gastric erosions. The inconsistency between the results of studies may be due to climate and atmospheric differences, factors relating to the management of antiparasitic plans, genetics, host sensitivity, and genetic differences in the parasite population.

Findings of another study performed on gastric lesions in Persian-Arabian horses revealed that the

frequency of gastric lesions in Persian-Arabian-trained horses was relatively high. However, the results of the aforementioned study reported a higher gastric lesion rate in non-glandular regions, compared to glandular areas. Moreover, in that study, horses with a history of long-term NSAID treatment had a higher frequency of gastric lesions. The above-mentioned study also suggested that horses with active training programs (excessive physical activities) indicated a higher frequency of gastric lesions (12). The results of the present study in several aspects, including the causes of gastric lesions, were consistent with those of the aforementioned study.

The results revealed a significant difference ($P<0.05$) between the blood indices of horses with normal stomachs and those of the horses with gastric erosions and granulomatous lesions, except for the MCHC and Lymph indices. Low levels of hematocrit, hemoglobin, and red blood cells in groups with lesions indicated mild anemia in the treatment group.

White blood cells and neutrophil count of the group with granulomatous lesions were higher, compared to the group with gastric erosion. However, there was no significant difference between the group with gastric erosion and the group with the gastric granulomatous lesion in terms of the Mono count (despite an increase, compared to the normal group). In addition, the significant increase in Eos count of the groups with erosion and granulomatous lesions in comparison with that of the normal group suggested the role of parasitic factors in causing gastric erosions and granulomatous lesions. However, the above-mentioned study (12) reported lower monocyte levels in horses with gastric erosion, compared to the group with normal horses.

Another study conducted on the frequency of gastric lesions in Caspian horses using endoscopic investigation suggested that NSAID drugs, lack of usage of antiparasitic drugs, and excessive physical activity as the primary causes of equine gastric lesions. Moreover, the mares had higher rates of gastric lesions, compared to the stallions. There was no significant difference between the hematological findings (blood parameters) of normal horses and those of horses with gastric lesions.

According to the above-mentioned study, 47.82% of the investigated horses indicated gastric lesions, and 81.8% of the lesions were located in the non-glandular region, while 18.2% of them were in the glandular section of the stomach (13). Since the current study focused on the lesions in the glandular region of the stomach, the lesion frequency was not comparable with those of the aforementioned studies.

In terms of appearance, the study that was performed on gastric lesions suggested erosions as shallow lesions with little or no bleeding on the mucosal layer and ulcers as penetrated lesions with or without bleeding. In terms of histology, loss of superficial mucosa was considered an erosion, while ulcer diagnosis depended on the lack of the entire mucosa layer. The muscularis mucosae layer was also exposed to the lesion (1).

Results of the present study did not report any gastric ulcer with muscularis mucosae involvement, and the lesions included gastric erosions and granulomatous lesions (ulcers without top-layer involvement). Findings of another study revealed that despite the significant, yet weak, relationship between the endoscopy severity scoring and depth of pathological ulcers, the endoscopy categorization of 57% of the non-glandular gastric ulcers as superficial ulcers was wrong. The endoscopist appeared to be able to determine the size of the ulcer better than its depth (14). It must be stated that pathology is more efficient in the determination of the depth of gastric lesions.

Another study investigated parasitic gastritis caused by habronemiasis. Its results indicated that the histopathology of separate lesions, such as diffuse infiltration of mononuclear inflammatory cells, along with infiltration of several eosinophils into the submucosa and mucosa layers of the stomach, was considered eosinophilic gastritis. Symptoms, including mucosal gastritis (catarrhal), diarrhea, progressive

weight loss, and ulcer, indicate gastric habronemiasis in horses (15).

The granulomatous gastritis of the present study caused by habronemiasis was consistent with the results of the mentioned study. The aforementioned results suggested chronic granulomatous lesions with thick connective walls and the existence of parasitic sections in the middle of the granulomas and infiltration of neutrophil, LYM, and macrophage inflammatory cells. In the current study, the expression of GDNF in gastric gland cells with erosions and granulomatous lesions was negative. However, there were GDNF-positive cells inside the normal gastric glandular cells.

Findings of the present research also suggest that the glial cell line-derived neurotrophic factor does not affect the pathogenesis of the equine gastric lesions (erosion and granulomatous). This neurotrophic factor mentioned by previous studies (16, 17) may also play a role in the development, differentiation, and organogenesis of various natural tissues. It is known that neuroendocrine cells are specialized mucous cells that play significant roles in the secretion of hormones, such as gastrin, somatostatin, and serotonin (2).

A study performed on the immunocytochemistry distribution of endocrine cells of the equine digestive system suggested various immunoreactive cells related to somatostatin, glucagon, and glisentin at the fundus and cardia regions of the equine stomach. However, most gastric immunoreactive cells are limited to the pylorus section of the stomach (18). Secretion of CgA depends on the cell type and the number of present cellular secretive granules (4). There are five primary types of neuroendocrine cells of gastric mucosa, including serotonin-secreting enterochromaffin cells, histamine-secreting enterochromaffin-like cells, somatostatin-secreting D-cells, and ghrelin-secreting P-cells (19).

The results of the CgA examination in the present study indicated that an increase in lesion severity leads to a significant increase in CgA-positive gastric glandular cells, while the number of CgA-positive cells in normal gastric glands was notably low. A study performed on enterochromaffin-like cells (ECL) in inductive gastric ulcers using acetic acid on rats reported the down-regulation of CgA in ECL cells of the margin of lesions. Accordingly, suppression of the activity of ECL cells may affect the duration of lesion healing and inhibition of gastric acid secretion (20).

Regarding prior studies, CgA plays a role in the adherence and proliferation of neuroendocrine cells. However, understanding the functions and mechanisms of CgA requires additional studies (21). Unlike the previous studies, the current study reported higher CgA expression in the margins of lesions and lower expression levels inside the lesion area. However, the CgA expression was significantly higher in gastric granulomatous lesions, compared to erosions (considering that the gastric surface was healthy).

Dendritic cells are considered primary antigen-presenting cells required for stimulation of antigen-specific T LYMs function. Accordingly, dendritic cells have a close relationship with the mechanism of T cells (22). Given the findings of the present study, the number of S100-positive dendritic cells among the gastric glands with granulomatous lesions was higher, compared to the other two groups. There was also a notable increase inside the connective tissue and muscles close to the granuloma, especially in the accumulation area of T cells, compared to the group with gastric erosion (most lesions indicated severe inflammation and a low number of T cells).

The P53 gene is a member of the onco-suppressors family that plays an important role in the cell cycle and apoptosis. The structure and function of p53 in cellular growth adjustment may have changed. The p53 gene changes and/or accumulation of abnormal p53 protein has been present in various observations, including renal, colon, lung, and gastric mucosa malignant tissues.

Studies have reported p53 gene mutations in carcinogenesis and pre-cancerous gastric lesions (23). Studies on the p53 gene expression during the recovery period of normal tissue in response to chronic epithelial

damage in pigs revealed that post-damage regeneration of normal tissues includes various cellular mechanisms. These mechanisms include A) selective localization of mitogenic growth factors promoting cell proliferation, B) simultaneous suppression of p53 that prevents cell proliferation, C) suppression of mitogenic growth factors after the healing of the damaged tissue, and D) high levels of p53 expression that help to decrease cell proliferation during the healing process (24).

Results of the present study suggested the notable increase of connective tissues in granuloma as the primary cause of increased p53 gene expression in gastric tissues with granulomatous lesions (compared to gastric erosions). Increased levels of p53 may control the cell proliferation rate in granulomatous lesions. However, the difference between the p53 expression of the normal group and that of the group with gastric erosion was not statistically significant. This finding may be due to the lower cell proliferation of gastric erosions.

An increase of p53-positive cells (p53 expression) in gastric granulomatous lesions can be considered a defense mechanism to protect the cells and a step toward healing the body. Moreover, an increased level of Ki67 expression indicates increased cell proliferation in groups with gastric lesions, compared to the group with a normal stomach. This finding may be a mechanism for cell proliferation for lesion healing. In addition, accompanied excessive increase in gene expression by genomic changes and undesirable p53 mutations can introduce neoplastic lesions in or close to the wounded area.

An investigation of the cancerous and precancerous lesions in rats revealed that induced gastritis caused by *Helicobacter pylori* in cancer patients was related to increased cell proliferation (Ki67) and apoptosis (TUNEL test), compared to the control group (25). Altogether, findings of the current study indicated that GDNF expression only occurred in healthy stomachs, and the protein expression in stomachs with erosions and granulomatous lesions was negative, which revealed that the factor does not affect the pathogenesis of the equine gastric lesions.

Another notable result of this study was that the level of CgA expression increased with the increased depth of gastric lesions (granulomatous lesions). This increase in CgA expression reveals the role that neuroendocrine cells play in the progression or pathogenesis of gastric lesions. The neuroendocrine cells have different secretions (such as gastrin, histamine, and somatostatin); therefore, differentiation of the cell type according to the equine gastric lesion requires additional research.

Given the increase in LYM cells (antigen-presenting cells to T LYMs) in granulomatous lesions, the increased S100 expression in dendritic cells that surround the granulomatous lesions of the glandular stomach was not significantly different from those of the other groups. In line with the previous findings, increased expression of Ki67 and p53 proteins in granulomatous lesions in comparison with other groups may be associated with the proliferation and control process of the cells in measures regarding the formation and healing of the lesion.

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Authors' Contribution

P. A.: participating in administrations, sampling and laboratory analyses, statistical analyses and interpretation of data as well as drafting the article; A. A.: Conception and design of the study, analysis and interpretation of data, final approval of the version to be submitted, Study design, participating in histopathological and immunohistological analyses, drafting the article or revising it critically for important intellectual content.

Ethics

This research has been conducted under the supervision of Research Ethics Committee of Islamic Azad University-Urmia Branch with approval ID (IR.IAU.URMIA.REC.1401.027) dated (2022-04-24).

Conflict of Interest

The authors declare that they have no conflict of interest.

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