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ORIGINAL ARTICLE

Age changes in the gastric mucosa of male albino rat: histological, immunohistochemical, histomorphometric and biochemical study

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ABSTRACT

Background: Age related changes in the stomach are associated with alterations in the structure and secretory function of the gastric glands. The present study aimed to investigate histological, histomorphometric and biochemical changes in the gastric mucosa of rats with age.

Materials and methods: Eighty adult male albino rats were randomly divided into four age groups, 20 rats in each (prepubertal, adolescent, adult, and senile). The gastric specimens were subjected to light microscopic examination using haematoxylin and eosin, PAS and Masson's trichrome stains. Immunohistochemical staining for caspase-3 and inducible nitric oxide synthase (iNOS) was carried out. Measurement of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) activity in gastric tissue homogenates was performed using ELISA. Quantitative analysis of vascular endothelial growth factor (VEGF) gene expression was done by real-time polymerase chain reaction (PCR).

Results: Light microscopic examination of gastric mucosa of senile rats revealed distortion of gastric glands and erosions. Surface mucous cells, mucous neck cells, parietal and chief cells exhibited cytoplasmic destruction, nuclear degeneration, apoptosis and oxidative damage.

There was a significant decrease in the mean gastric mucosal thickness, increase in collagen content and decrease in mucous content with the advance of age. These morphological changes were associated with a significant decrease in SOD and GPx activity and increase in MDA activity, in addition to decreased VEGF gene expression.

Conclusions: Gastric mucosa of aged rats showed histological and immunohistochemical alterations. These changes were associated with oxidative stress, decreased antioxidant capacity and decreased angiogenesis.

Keywords: age, gastric mucosa, rats, antioxidant, angiogensis

INTRODUCTION

Aging is a biological process that causes loss of tissue and organ function. Damage of DNA, telomere shortening, production of high amount of reactive oxygen species (ROS), abnormal gene activities, metabolic changes, mitochondrial dysfunction are among the changes detected in ageing cells [13].

All organs are deeply affected by aging process. Stomach is among these organs undergoing physiological and pathological alterations during aging. Clinical and experimental studies have reported aging-related changes including decrease in bicarbonate secretion, gastric blood flow rate, delay in gastric emptying and mucosal changes related with gastric morphology and function [28].

Aging gastropathy is an important and clinically relevant issue for aging world population due to prolonged life span. Older patients have much greater risk of gastroduodenal ulcers and gastrointestinal complications (e.g., non-steroidal anti-inflammatory drugs (NSAIDS)-induced gastric injury) than younger patients [29]. Moreover, aging of the stomach is associated with increased incidence of gastric cancer, which ranks the fifth most common cancer and the third leading cause of cancer-related deaths worldwide [20].

Free radicals including singlet oxygen, superoxide ion and hydrogen peroxide are critical to the aging process. Within the cell they can distort the structure of molecules, abolish their function create a molecular imbalance which can be observed at biomolecular level [5].

In the reviewed literature, studies concerning the effect of aging on the gastric mucosa was limited to two age groups. The present study was designed to demonstrate histological and histomorphometric and biochemical changes that occur in the gastric mucosa of rats of different ages.

MATERIALS AND METHODS

Animals

The present study was carried out on 80 male albino rats, which were obtained from the Animal House, Faculty of Medicine, Cairo University following the guidelines for the care and use of laboratory animals (approval NO. CU-III-F-9-23). They were housed in cages, five rats/cage and allowed to standardized laboratory diet and water ad libitum throughout the experiment. The animals were divided into four age groups (20 rats in each) as follows:

- group I (prepubertal): Aged from 1 to 1.5 months [27],
- group II (adolescent): Aged from 2 to 3 months [27],
- group III (adult): Aged from 9 to 18 months [25],
- group IV (senile): Aged from 20 to 24 months [25].

Methods

All animals were anaesthetized by mild ether inhalation and sacrificed by cervical dislocation, then specimens of the glandular stomach (corpus or pars glandularis) were subjected for the following:

Histological study

The specimens were dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin and sagittal sections of 5 μm thickness were cut and stained by:

1) **Haematoxylin and eosin (H&E):** For routine histological examination [11].
2) **Masson's trichrome:** For detection of collagen content [7].
3) **Periodic acid Schiff (PAS):** For detection of glycoproteins [7].
4) **Immunohistochemical staining for caspase-3 antibody [6]:** Primary antibody: Anti-caspase-3 mouse monoclonal antibody (Dako company, Cairo, Egypt, Catalog No. IMG-144A at a dilution 1/200). For detection of apoptosis.
5) **Immunohistochemical staining for inducible nitric oxide synthase (iNOS) [15]:** Primary antibody: rabbit polyclonal Inos antibody (DAKO Corporation, Carpinteria, CA, USA at a dilution 1/200). For detection of oxidative stress.

Histomorphometric stud

Five non overlapping fields from five sections (from each rat) of 20 rats per group were randomly chosen. The measurements were obtained using "Leica Qwin 500 C" image

analyzer computer system Ltd. (Cambridge, England). The following parameters were measured:

1) Thickness of gastric mucosa in H&E-stained sections at a magnification 100×. Four measurements were taken and the average was calculated for each slide.
2) Area percent of collagen fibers in Masson's trichrome stained sections at a magnification 400×.
3) Optical density of mucous content in PAS-stained sections at a magnification 400×.
4) Area percent of caspase-3 immuno-expression in the gastric tissue at a magnification 400×.
5) Area percent of iNOS immuno-expression in the gastric tissue at a magnification 400×.
6) Area percent of collagen fibers, optical density of glycogen, area percent of caspase-3 and iNOS immuno-expression were done by transforming the colored images into grey images then masking the positive areas by a red binary color using image analyzer software.

Biochemical study

1) **ELISA:** The gastric tissue homogenate was prepared for assessment of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) by an ELISA kit as described in manufacturer's instructions (Biospes Co. Chongqing, China) and lipid peroxidation marker malondialdehyde (MDA) by an ELISA kit as described in manufacturer's instructions (Biodiagnotics Co., Upton upon Severn, UK).
2) **Quantitative analysis of VEGF gene by real time polymerase chain reaction (RT-PCR):** Real time PCR was done to detect expression of VEGF gene for the evaluation of angiogenesis. Extraction of RNA was done by homogenization in TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the instructions of the manufacturer. The RNA concentrations and purity were measured with an ultraviolet spectrophotometer [33].

Complementary DNA (cDNA) synthesis: The cDNA was synthesized from 1 ug RNA using Superscript III First-Strand Synthesis; a system described in the manufacturer's protocol (Invitrogen, Life Technologies). One ug of total RNA was mixed with 50 μM oligo (DT) 20, 50 ng/μL random primers, and 10 mM dNTP mix in a total volume of 10 μL. The mixture was incubated at 56°C for five minutes. and then placed on ice for three minutes. The reverse transcriptase master mix containing 2 μL of 10× RT buffer, 4 μL of 25 mM MgCl2, 2 μL of

0.1 M DTT, and 1 μL of SuperScript III RT (200 U/μL) was added to the mixture and was incubated at 25°C for ten minutes followed by 50 minutes at 50°C.

Real time quantitative PCR: Quantitative RT-PCR was performed in a 25-μL reaction volume consisting of 2X SYBR Green PCR. Master Mix (Applied Biosystems), 900 nM of each primer and 2–3 μL of cDNA. Amplification conditions were 2 min. at 50°C, 10 min. at 95°C and 40 cycles of denaturation for 15 seconds and annealing/extension at 60°C for 10 min. Data from real-time assays were calculated using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalized to the beta actin gene and reported as fold change..

The primer sequence of the VEGF was:

F: 5′-GAGGAGTTCAACATCGCCAT-3′

R: 5′-GAGGAGTTCAACATCGCCA-3′

Statistical analysis: Numerical data were analyzed using the Statistical Package for Social Science (SPSS) version 21. The mean value and standard deviation (SD) were calculated. One way ANOVA (Analysis of variance) was performed to test the difference between groups as regards mean values of measured variables; Tukey (Post hoc test) was used for multiple comparisons between pairs of groups. For interpretation of results of tests of significance, significance was accepted at $p \leq 0.05$.

RESULTS

A. Light microscopic results:

Group I (prepubertal) and II (adolescent): Histological examination of sections of the gastric mucosa from prepubertal and adolescent rats showed similar findings. In H&E-stained sections, the gastric mucosa was formed of surface epithelium and tightly packed simple tubular or branched tubular gastric glands limited by muscularis mucosae. The gastric glands were perpendicular to the surface and were composed of isthmus, neck and base (Fig. 1A). They opened on the surface by short and narrow gastric pits distended with mucus secretion (Figs. 1A, B). The submucosa contained blood vessels (Fig. 1A). The gastric mucosal epithelium was formed of surface mucous cells, which showed oval basal nuclei surrounded by clear cytoplasm. The neck region of the gastric gland was lined by low columnar mucous cells with basal rounded nuclei and pale foamy cytoplasm (Fig. 1B). Parietal (oxyntic) cells were spherical or pyramidal in shape with acidophilic cytoplasm and central rounded nuclei (Fig. 1C). Peptic (chief) cells were recognized by their deep basophilic cytoplasm and basally situated rounded nuclei (Fig. 1C). Masson's trichrome-stained sections revealed few thin collagen fibers between gastric glands as well is in the submucosa (Fig. 1D). In PAS-stained sections, a strong positive reaction was observed in surface mucous cells as well as mucous neck cells (Fig. 1E).

Group III (adult): Section stained by H&E showed apparently normal histology of gastric mucosa. Mild leucocytic infiltration was observed in the submucosa (Fig. 2A). The surface mucous cells and mucous neck cells showed normal histology. Pyknotic nuclei of mucous neck cells were occasionally seen (Fig. 2B). Apparently normal parietal cells and chief cells were observed. Few chief cells exhibited patchy destruction of cytoplasm, shrunken nuclei and karyolysis (Fig. 2C). Masson's trichrome-stained sections showed moderate increase in the amount of collagen fibers in the submucosa (Fig. 2D). Sections stained with PAS revealed moderate reaction in the surface mucus cells (Fig. 2E).

Group IV (senile): The gastric glands of this group presented a wide range of histological alterations. Haematoxylin and eosin-stained sections exhibited gastric erosions in the surface epithelium and extravasation of blood between gastric glands. (Fig. 3A). A characteristic of this age group was the heterogenicity of histological patterns among different specimens. In some specimens, cytoplasmic vacuolations were encountered in the isthmus region of the glands (Fig. 3A). Other specimens showed distorted architecture with dilatation of the lumina in the middle and basal regions of the glands (Fig. 3B). Diffuse leukocytic infiltration between the gastric glands and in the submucosa was also detected in this group (Figs. 3A, B). Surface mucous cells and mucous neck cells exhibited cytoplasmic vacuoles and patchy destruction of the cytoplasm. Different forms of nuclear degeneration such as pyknonosis, karolysis (ghost nuclei) and karyorrhexis were encountered. Nuclear dysplasia was also detected in the form of nuclear hypertrophy and bizarre shaped nuclei. Some glands showed apoptotic-like cells with deeply acidophilic cytoplasm and condensed nuclear chromatin (Fig. 3C).

Degenerative changes were also observed in parietal and chief cells, which showed lightly stained degenerated cytoplasm with loss of inter-cellular boundaries. Apoptotic-like cells, nuclear pyknosis, pleomorphism, bizarre-shaped nuclei and peri-nuclear vacuolation were seen (Fig. 3D). Masson's trichrome-stained sections showed an apparent increase in the amount of collagen fibers surrounding gastric glands and, in the submucosa, (Fig. 3E). Sections stained with PAS revealed weak reaction of the surface epithelium (Fig. 3F).

B. Immunohistochemical results:

Group I (prepubertal) and II (adolescent): In the two groups, weak immunoreactivity of caspase-3 and iNOS-stained sections was observed in the gastric glands (Figs. 4A, B).

Group III (adult): Caspase-3 and iNOS-stained sections showed moderate immunoreactivity in the gastric glands (Figs. 4C, D).

Group IV (senile): Strong immunoreactivity of the gastric glands was observed in caspase-3 and iNOS-stained sections (Figs. 4E, F).

C. Histomorphometric results:

Thickness of gastric mucosa in Hx & E-stained sections: There was a statistically significant age-related decrease in gastric mucosal thickness in senile rats as compared to prepubertal rats, adolescent, and adult rats ($p < 0.001$* in the three groups) (Table 1) (Graph 1). Gastric mucosal thickness decreased in adolescent rats as compared to prepubertal rats; however, this difference was statistically non-significant ($p = 0.062$). In adult rats, there was a

significant age-related decrease in gastric mucosal thickness as compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*) (Table 1) (Fig. 5A).

Area percent of collagen fibers in Masson's trichrome-stained sections: The collagen area percent in the peri-glandular and submucosal regions increased in senile rats as compared to prepubertal, adolescent and adult rats. This increase was found to be statistically significant when compared to prepubertal rats ($p < 0.001$*) and adolescent rats ($p < 0.001$*), however, it was non-significant when compared to adult rats ($p = 0.209$) (Table 2) (Graph 2). In adolescent rats, there was a statistically non-significant increase in collagen area percent as compared to prepubertal rats ($p = 0.757$). Regarding adult rats, there was a significant increase in collagen area percent as compared to adolescent and prepubertal rats ($p = 0.032$* and $p = 0.001$* respectively) (Table 1) (Fig. 5B).

Optical density of mucous content in PAS-stained sections: There was a statistically significant decrease in the mucous content of the mucous cells in the gastric glands of senile rats as compared to prepubertal, adolescent and adult rats ($p < 0.001$* in the three groups) (Table 3) (Graph 3). A non-significant decrease in the mucous content of gastric glands in adolescent rats was found as compared to prepubertal rats ($p = 1.000$). In adult rats, there was a significant decrease in mucous secretion as compared to adolescent and prepubertal rats ($p = 0.011$* and $p = 0.001$* respectively) (Table 1) (Fig. 5C).

Area percentage of caspase-3 immunoexpression in the gastric tissue: There was a statistically significant increase in the area percent of caspase-3 immunoexpression in the gastric homogenates of senile rats in comparison to prepubertal, adolescent and adult rats ($p < 0.001$* in the three groups) (Table 4) (Graph 4). In adolescent rats, there was a statistically non-significant increase in area percent as compared to prepubertal rats ($p = 0.097$). Gastric glands of adult rats showed a significant increase in caspase-3 immunoexpression when compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*) (Table 1) (Fig. 5D).

Area percentage of iNOS immuno-expression in the gastric tissue: Expression of iNOS in gastric tissue increased significantly in senile rats compared to prepubertal, adolescent and adult rats ($p < 0.001$*, $p < 0.001$*, $p = 0.012$* respectively) (Table 5) (Graph 5). A non-significant increase in iNOS immuno-expression in adolescent rats was found as compared to prepubertal rats ($p = 0.070$). In adult rats, there was a significant increase in iNOS immuno-expression as compared to adolescent rats ($p < 0.001$*) and prepubertal rats ($p < 0.001$*) (Table 1) (Fig. 5D).

D. Biochemical results:

Superoxide dismutase: The gastric homogenates of gastric mucosal specimens of senile rats demonstrated a statistically significant decrease in the mean SOD level as compared to prepubertal ($p < 0.001$*), adolescent ($p < 0.001$*) and adult rats ($p = 0.026$*) (Table 6) (Graph 6). When comparing adolescent rats to prepubertal rats, a non-significant decrease was observed ($p = 1.000$). In adult rats, there was a significant decrease in the mean SOD level as compared to adolescent rats ($p = 0.034$*) and prepubertal rats ($p = 0.013$*) (Table 2) (Fig. 6A).

Glutathione peroxidase: There was an age-related decrease in the mean GPx level in the gastric homogenate of senile rats as compared to prepubertal, adolescent and adult rats ($p < 0.001$* in the three groups). Gastric mucosa of adult rats showed a significant decrease in the mean GPx level as compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < .001$*). When comparing adolescent rats to prepubertal rats, a non-significant decrease in the mean GPx level was observed ($p = 0.188$) (Table 2) (Fig. 6B).

Malondialdehyde: The senile rats showed a statistically significant increase in the mean MDA level in the gastric tissue in comparison to prepubertal, adolescent and adult rats ($p < 0.001$* in the three groups). Adult rats showed a highly significant increase in the mean MDA level as compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*). A non-significant increase in the mean MDA level was observed in adolescent rats compared to prepubertal rats ($p = 1.000$) (Table 2) (Fig. 6C).

Vascular endothelial growth factor: The mean value of VEGF gene expression in the gastric tissue of senile rats showed a statistically significant decrease compared to prepubertal adolescent and adult rats ($p < 0.001$* in the three groups). Tissue samples from adult rats revealed a significant decrease in the mean VEGF gene expression as compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*). In adolescent rats, a non-significant decrease in VEGF gene expression was found as compared to prepubertal rats ($p = 0.062$*) (Table 2) (Fig. 6D).

DISCUSSION

The present study examined age changes in gastric mucosa of prepubertal, adolescent, adult and senile rats via light microscopic, immunohistochemical and biochemical techniques.

In the current work, histological examination of gastric mucosa of prepubertal and adolescent rats showed normal histology. There was a non-significant decrease in gastric mucosal thickness and mucous content, in addition to non-significant increase in collagen in the gastric

mucosa of adolescent rats as compared to prepubertal rats. In adult rats, despite of mild morphological changes, there was a statistically significant increase in collagen fibers, a decrease in mucus content as well as gastric mucosal thickness in this group as compared to prepubertal and adolescent rats. In contrary, El-Shall [12] reported strong positive PAS reaction in surface and mucous neck cells in 18 months old rats indicating unaffected mucus secretion in this age group. The difference in the finding between the later author and the current work might be due to the difference in the region of the stomach used in her study, which was the fundus of the stomach.

Gastric tissue obtained from adult rats in the present study showed few cells with pyknotic nuclei and destructed cytoplasmic. This finding was associated with moderate caspase 3 immunoexpression, which was confirmed by the statistically significant increase in caspase-3 expression as compared to prepubertal and adolescent rats. In a contradictory study, Akbulut et al. [4] found that there was no significant difference in caspase-3 immunoreactivity of gastric mucosa between young and middle-aged rats.

Oxidative stress was observed in the gastric mucosa of adult rats in the present study as there was a significant increase in iNOS immunoexpression in the gastric mucosa of this group compared to prepubertal and adolescent groups. This was accompanied by a significant decrease in the level of antioxidant enzymes SOD and GPx and a significant increase in the level of oxidative stress marker MDA in adult rats compared to the young once. Similarly, Vucevic et al. [31] found that MDA level in gastric tissue of 18 months old rats was significantly higher than in three months old rats. The authors attributed this increase to the ROS generated by the synthesis of eicosanoids, which are believed to be among the major sources of ROS in the aging process.

Examination of gastric tissue from senile group in the current work revealed evident histological changes including distortion of gastric glands, nuclear and cytoplasmic degeneration, marked congestion of blood vessels, increased collagen deposition and decreased mucous content. These results were consistent with the data provided by Lutnicki et al. [21], who reported lack of organization of glandular structures, different size and stain ability of cell nuclei, increase in connective tissue and thickening of blood vessel walls. In a contradictory study, Kirmızikan and Esrefoglu [18] demonstrated that microscopical features of aged stomachs were as normal as young ones except increased vascularization and congestion especially at the bottom of the glands.

Fibrosis of gastric mucosa of senile rats observed in the present study was confirmed by the significant increase collagen area percent in the peri-glandular and submucosal regions

compared to prepubertal, adolescent and adult groups. Similar findings were observed by El-Shall [12] and Ait-Belkacem et al. [3], who pointed out that networks of collagen fibers increased significantly with advancing age. However, the significant increase in collagen area percent of gastric tissue found in the current work was associated with a significant decrease in gastric mucosal thickness indicating atrophy of gastric glands. Similarly, Tarnawski et al. [29] demonstrated partial atrophy of gastric glands accompanied by a significant increase in connective tissue replacing glandular cells in aging rats. On the other hand, Brito et al. [8] reported that no alterations of collagen fibers have been found in the gastric mucosa of old rats. The difference in the results between those authors and the present study might be due to the difference in the classification of age groups as the authors considered the 18 months old rats as the senile group, while the current study considered them as adult group. Thinned gastric mucosa with atrophy in the basal layer of the glands in senile rats was also observed by Lutnicki et al. [21]. Furthermore, Kirmizikan and Esrefoglu [18] reported an obvious decrease in the thickness of gastric mucosa in aged rats compared to young ones. Those authors pointed out that insufficient secretion of prostaglandins and bicarbonate results in a decrease in the thickness of mucus layer is naturally associated with decreased number of glandular cells including glandular mucous. On the contrary to the current findings, Majumdar et al. [23] reported an increase in overall thickness of the gastric mucosa in 24 months old rats when compared to four months old animals and attributed that to massive collagen deposition between the glands and the muscularis mucosa.

Sloughing of surface epithelium and gastric erosions were detected in gastric specimens obtained from senile rats in the present study, which were consistent with the work carried out by El-Shall [12]. The authors attributed the increased incidence of fundic ulcer observed in the senile group to increased susceptibility of the mucosa to various damaging agents together with impediment of the repair process. On the other hand, no macroscopic ulcers were detected in the stomach of rats at any age as evidenced by Marmol et al. [24].

In the present study, atrophy of gastric tissue was accompanied by a statistically significant decrease in mucus secretion in senile rats compared to prepubertal, adolescent and adult rats. These results agreed with El-Shall [12], who reported a decrease in PAS reaction in mucous neck cells in 24 months old rats. Those authors attributed that to decreased secretory activity of the gastric mucosal cells with decreased number of intracytoplasmic secretory granules at the ultrastructural level. In a recent study carried out by Kirmizikan and Esrefoglu [18], weak positive PAS-stained mucous neck cells were associated with decreased luminal mucus layer thickness in aged rat stomachs compared to young ones. The author explained these findings

by the decrease in prostaglandins which stimulate gastric mucus and bicarbonate secretion as well as relative loss of glandular cells with subsequent thinning of surface mucus layer indicating poor mucosal barrier in aged animals.

In the present work, inflammation of gastric mucosa with age was observed in adult and senile groups as evident by leucocytic infiltration between gastric glands and in the submucosa. However, Kang et al. [7] pointed out that there was no increase of inflammatory cell infiltration depending on age**.**

In the present work, histological examination of gastric specimens obtained senile rats showed apoptotic-like cells with deeply acidophilic cytoplasm and condensed nuclear chromatin. This was confirmed by the significant increase in caspase-3 immunoexpression in the gastric tissue of senile rats as compared to prepubertal, adolescent and adult rats. These data agreed with Tarnawski et el. [30], who found a significant increase in proapoptotic cleaved caspase-3 and a reduction of antiapoptotic surviving in the gastric mucosa of aged rats. Those authors clarified that the imbalance between proapoptotic and antiapoptotic factors results in increased apoptosis and partial gastric mucosal atrophy in aging rats which also provides an explanation for its increased susceptibility to injury. In accordance with the current study, increased caspase-3 activity in very old rats have been also reported by Zhang et al. [32] and Akbulut et al. [4]. Lee et al. [19] attributed apoptosis to increased ROS and decreased antioxidants level in aged animals.

In the current study, there was a significant age-related increase in the immunoexpression of iNOS in the gastric mucosa of senile rats as compared to adult, adolescent and prepubertal rats indicating oxidative damage. This finding was associated with a significant decrease in the level of SOD and GPx enzymes and a significant increase in MDA in senile rats compared to other groups. Similar findings were detected by Lutinicki et al. [21], who reported a statistically significant decrease in the activity of SOD and GPx enzymes in the stomach of old rats. Furthermore, Marmol et al. [24] found an evidenced increase in lipid peroxides together with a simultaneous decrease in antioxidant enzymes SOD and catalase in the stomach of 24 months old rats. It was reported that the balance between the generation of oxygen free radicals and the activity of antioxidative system can play a significant role in the aging processes [10]. Superoxide dismutase and GPx protect cells and tissues from oxidative damage in the frame of enzymatic antioxidative system, but in several cases, the system becomes insufficient to neutralize excessively generated reactive oxygen forms which play an important role in the pathogenesis of gastric mucosal damage [9].

One of the best-validated signalling pathways in angiogenesis is VEGF and its receptors [26]. Angiogenesis is initiated and regulated by angiogenic growth factors, e.g., VEGF, a fundamental stimulator of angiogenesis that acts on endothelial cells, which are the key targets and effectors of angiogenesis [14].

In the current study, an age-related significant decrease in VEGF expression was observed in gastric tissue of adult and senile groups compared to young ones. This was in accordance with Ahluwalia et al. [2] and Tarnawski et al. [29], who found an age-related reduction in VEGF expression and impaired angiogenesis in the gastric mucosa of aging rats.

It was reported that hypoxia is a potent stimulus for VEGF gene activation [16], therefore, it could be expected that aging gastric mucosa might have increased VEGF expression. However, Ahluwalia et al. [1] demonstrated that aging gastric mucosa did not express higher VEGF levels despite increased tissue hypoxia, indicating that gastric mucosa of aged rats loses its sensitivity to hypoxia at least with respect to VEGF gene activation. The authors clarified that the reduced VEGF levels in aging gastric mucosa inhibits angiogenesis and delays healing of injured gastric mucosa. This might explain the presence of gastric ulcers and erosions in the gastric mucosa of senile rats in the current work.

Age-related deficiency in mucosal repair secondary to reduced VEGF expression in the stomach of senile rats was also reported by Majumdar [22] and Kang et al. [7]. The later authors reported that VEGF expression was significantly decreased in 18 months old and two years old rats compared to the seven months old rats.

CONCLUSIONS AND RECOMMENDATION

In conclusion, there is evidence that the gastric mucosa undergoes morphological changes with age. These alterations were associated with increased oxidative stress markers and deficient antioxidant capacity as well as decreased angiogenesis. The findings of the current work might be correlated with increased incidence of gastritis, gastric erosions and gastric tumours in elderly population. Administration of antioxidants might decelerate age related deterioration in gastric mucosal function.

ARTICLE INFORMATION AND DECLARAITONS

Data availability statement

Data are available upon reasonable request from the corresponding author.

Ethics statement

The study was done following the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and the guidelines for the care and use of laboratory animals (approval NO. CU-III-F-9-23).

Author contributions

Dr. Shereen Abdel Fattah Mohammed: conceptualization of the idea of the research. Dr. Ayman Abo El-Enein Rizk: supervision and guiding the authors. Dr. Mogeda Mahdy Nasralla: supervision and guiding the authors. Dr. Doaa Mahmoud Shuaib: writing the paper and data analysis. Dr. Marwa Mahmoud Elsayed: performed the experiment + statical analysis.

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Conflict of interest

The authors declared no conflict of interest.

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Table 1. Mean values of gastric mucosal thickness, area percent of collagen fibers, optical density of mucous content, area percent of caspase-3 and iNOS immuno-expression all rat grups.

Groups	Gastric mucosal thickness (μm) (Mean ± SD)	Area percent of collagen fibers (Mean ± SD)	Optical density of mucous content (Mean ± SD)	Area percent of caspase-3 immuno-expression (Mean ± SD)	Area percenta of iNOS immu expression (Mean ± SD
(Prepubertal)	525 ± 95.6	0.60 ± 0.54	65.04 ± 1.5	0.898 ± 0.213	0.87 ± 0.329
I (Adolescent)	457 ± 82.4	3.4 ± 2.1	61.6 ± 1.1	1.76 ± 0.439	2.82 ± 0.554
III (Adult)	365 ± 74.6	9 ± 4.1	50.3 ± 3.4	8.88 ± 0.719	12 ± 1.185
IV (Senile)	234 ± 56.3	15 ± 3.5	29.1 ± 3.9	14.9 ± 1.29	14.5 ± 1.688

Table 2. Mean values of SOD, GPx, MDA and VEGF gene expression in the gastric tissue of all rat groups.

Groups	SOD (Mean ± SD)	GPx (Mean ± SD)	MDA (Mean ± SD)	VEGF gene expression (Mean ± SD)
I (Prepubertal)	28.68 ± 2.4	88 ± 1.5	65.56 ± 2.5	6.5 ± 0.27
II (Adolescent)	27.64 ± 4.1	84.3 ± 0.9	63.7 ± 3.9	5.7 ± 0.54
III (Adult)	20.18 ± 2.8	67 ± 4.4	87 ± 5.4	3.2 ± 0.43
IV (Senile)	12.42 ± 4.7	41 ± 1.9	127.1 ± 7.4	1.04 ± 0.04

GPx — Glutathione peroxidase; MDA — Malondialdehyde; SOD — Superoxide dismutase; VEGF — Vascular endothelial growth factor

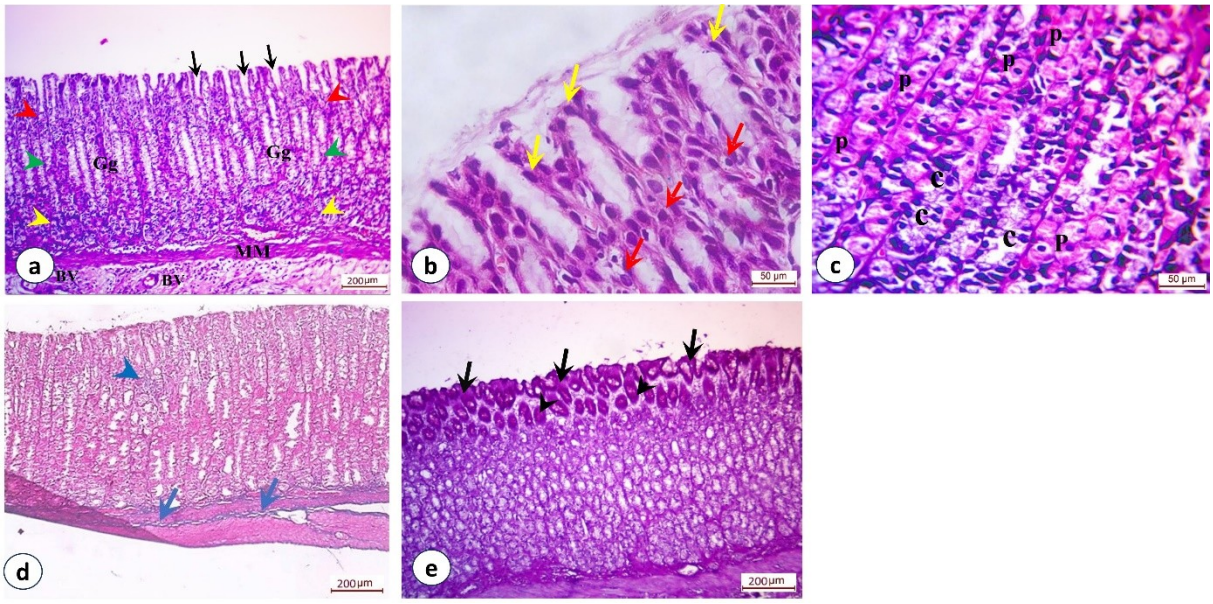

Figure 1. Photomicrographs of sections of the gastric mucosa of rats from group I (prepubertal group): **A.** Surface epithelium is invaginated by gastric pits (arrows). Gg reach down to MM. The isthmus (red arrowheads), neck (green arrowheads) and base (yellow arrowheads) of the glands are demonstrated. The submucosa shows BV (Hx&E×100). **B.** Surface mucous cells have clear cytoplasm and basal oval nuclei (yellow arrows). Mucous neck cells exhibit pale foamy cytoplasm and rounded nuclei (red arrows) (Hx&E×400). **C.** Parietal cells (P) show deep acidophilic cytoplasm and central rounded nuclei. Chief cells (C)

are seen with basophilic cytoplasm and basal rounded nuclei (Hx&E×400). **D.** Few collagen fibers between gastric glands (arrowhead) and in the submucosa (arrows) are illustrated (Masson's trichrome ×100). **E.** Strong positive PAS reaction is shown in the surface epithelium (arrows) and in the mucus neck cells (arrowheads) (PAS ×100). Abbreviations: BV — blood vessels; Gg — gastric glands; H&E — haematoxylin and eosin; MM — muscularis mucosa.

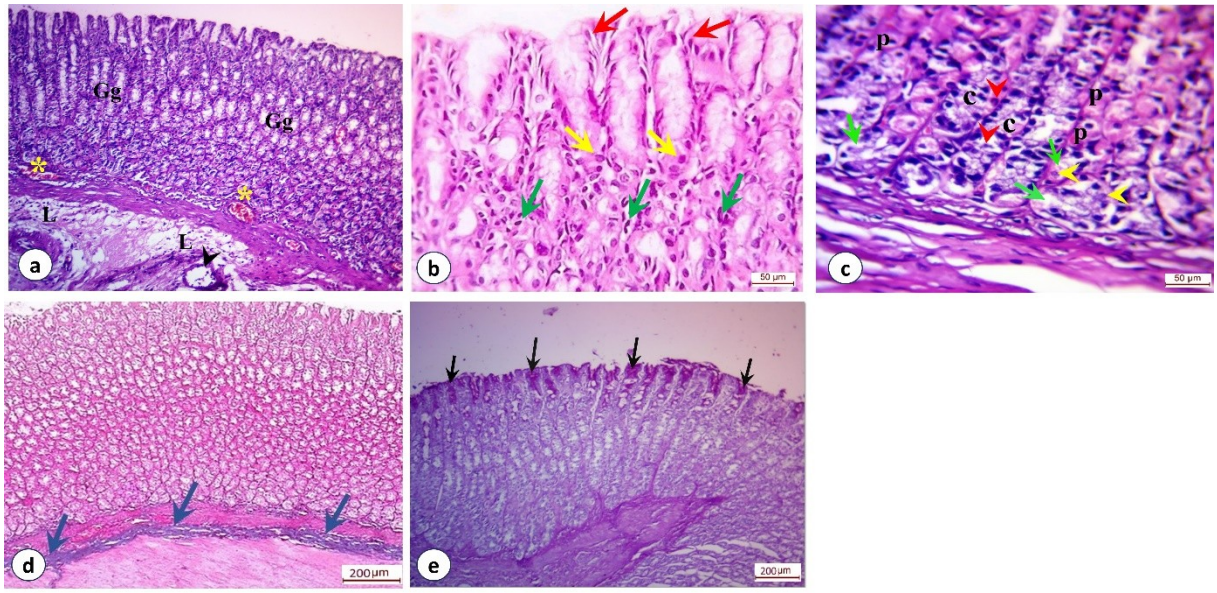

Figure 2. Photomicrographs of sections of the gastric mucosa of rats from group III (adult): **A.** Apparently normal Gg are illusrated. Note blood vessels (*) at the basal part of the glands. The submucosa shows mild leucocytic infiltration (L). Blood vessel in the submucosa (arrowhead) is demonstatred (Hx&E×100). **B.** Surface mucous cells (red arrows) and muscus neck cells (yellow arrows) are apparently normal except for few pyknotic nuclei (green arrows) (Hx&E×400). **C.** Apparently normal parietal cells (P) and chief cells (C) are demonstaterd. Few chief cells exhibit patchy destruction of cytoplasm (green arrows), shrunken nuclei (red arrowheads) and karyolysis (yellow arrowheads) (Hx&E×400). **D.** Moderate amount of collagen fibers are seen in the submucosa (arrows) (Masson's trichrome

×100). **E.** The surface epithelium exhibits moderate PAS reaction (arrows) (PAS ×100). Gg — gastric glands; H&E — haematoxylin and eosin; PAS — Periodic acid Schiff.

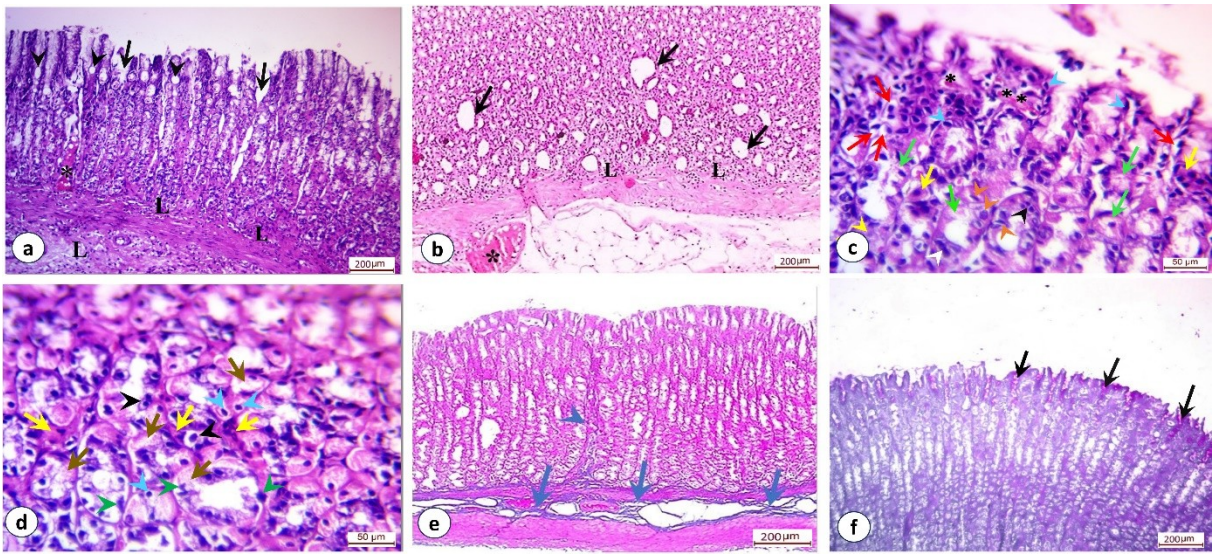

Figure 3. Photomicrographs of sections of the gastric mucosa of rats from group IV (senile): **A.** Erosions of the gastric mucosa (arrows) and cytoplasmic vacuolations (arrowheads) are illustrated in the isthmus region of the glands. Leucocytic infiltration (L) is demonstrated at the bases of the glands and in the submucosa. Note extravasation of blood (*) between gastric glands (Hx&E×100). **B.** The lumina of gastric glands are widened (arrows) with marked leucocytic infiltration (L) in the basal part of the glands. Blood vessel (*) is demonstrated in the submucosa (Hx&E×100). **C.** Mucous cells shows patchy destruction of cytoplasm (green arrows) and cytoplasmic vacuolation (red arrows). Nuclear pyknosis (blue arrowheads), karyorrhexis (black arrowhead), karyolysis (yellow arrowhead), nuclear hypertrophy (white arrowhead) and bizarre-shaped nuclei (orange arrowheads) are shown. Note apoptotic-like cells (yellow arrows) and extravasation of blood (*) (Hx&E×400). **D.** Parietal and chief cells show degenerated cytoplasm (brown arrows), pyknotic nuclei (blue arrowheads), peri-nuclear vacuolation (black arrowheads) and nuclear pleomorphism (green arrowheads). Apoptotic like

cells (yellow arrows) are illustrated (Hx&E×400). **E.** Moderate amount of collagen fibers between gastric glands (arrowhead) and abandunt collagen fibers in the submucosa (arrows) are shown. **F.** Weak PAS reaction is seen in the surface epithelium (arrows). H&E — haematoxylin and eosin; PAS — Periodic acid Schiff.

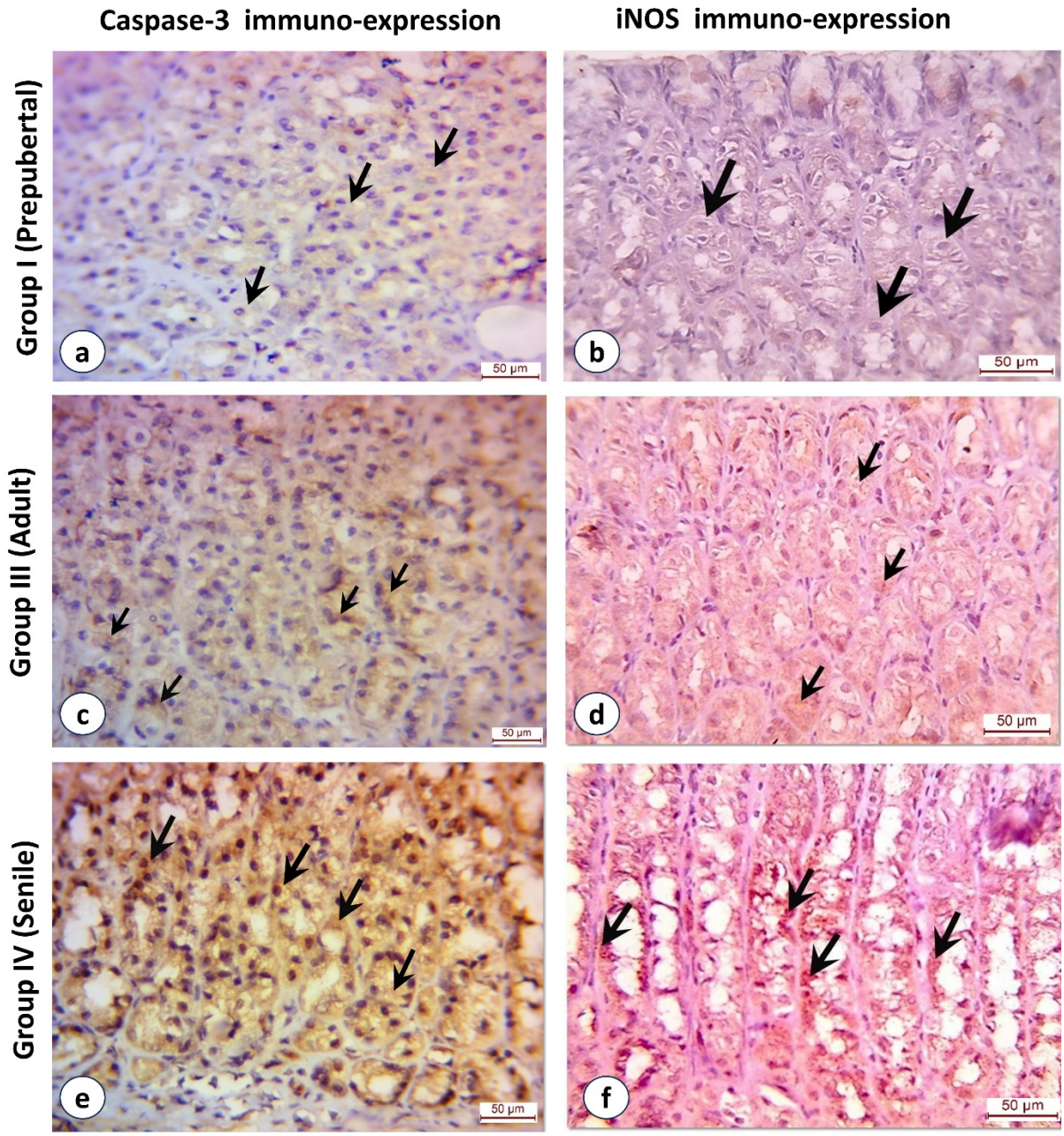

Figure 4. A photomicrograph rat gastric mucosa: **A.** Group I (prepubertal group) shows weak caspase-3 immunoreaction in the gastric glands (arrows) (Caspase-3×400). **B.** Section from

group I (prepubertal group) demonstrates weak inducible nitric oxide synthase (iNOS) immunoreactivity in the gastric glands (arrows) (iNOS×400). **C.** In group III (adult group), moderate caspase-3 immunoreaction (arrows) is shown in the gastric glands (Caspase-3×400). **D.** Group III (adult group) reveals moderate iNOS immunoreaction in the gastric glands (arrows) (iNOS×400). **E.** Group IV (senile group) exhibits moderate caspase-3 immunoreaction in the gastric glands (arrows) (Caspase-3×400). **F.** Group IV (senile group) reveals strong iNOS immunoreactivity in the gastric glands (arrows) (iNOS×400).

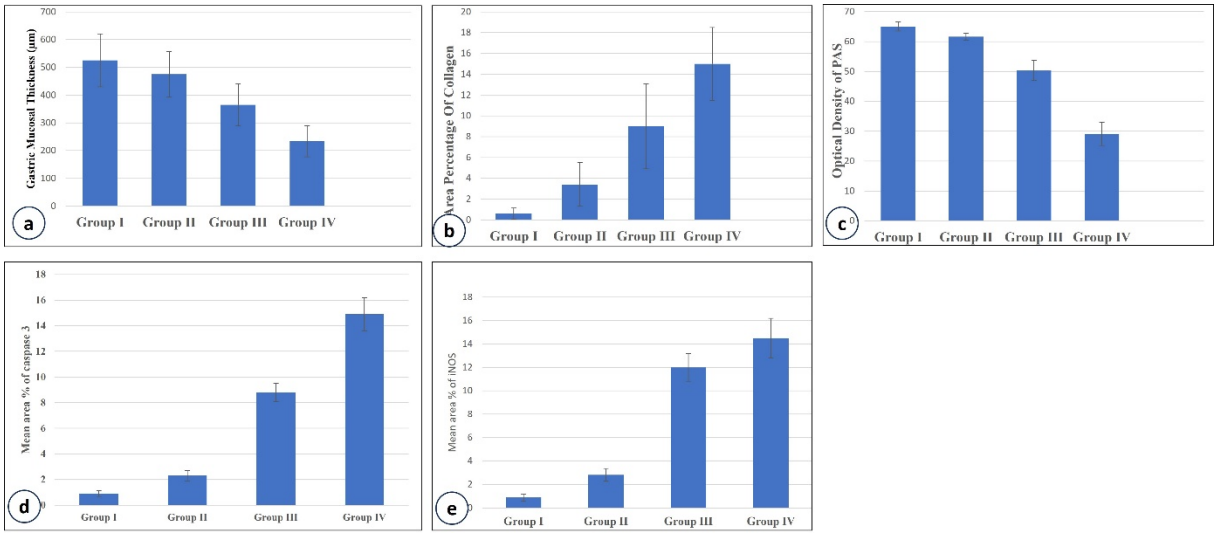

Figure 5. The mean (error bars: ± standard deviation); **A.** Gastric mucosal thickness (µm) in the studied groups; **B.** Area percent of collagen in Masson's trichrome-stained sections in the studied groups; **C.** Optical density of PAS-stained sections in the studied groups; **D.** Area percent of caspase-3 immunoexpressioin in the studied groups; **E.** Area percent of iNOS immunoexpressioin in the studied groups. iNOS — inducible nitric oxide synthase; PAS — Periodic acid Schiff.

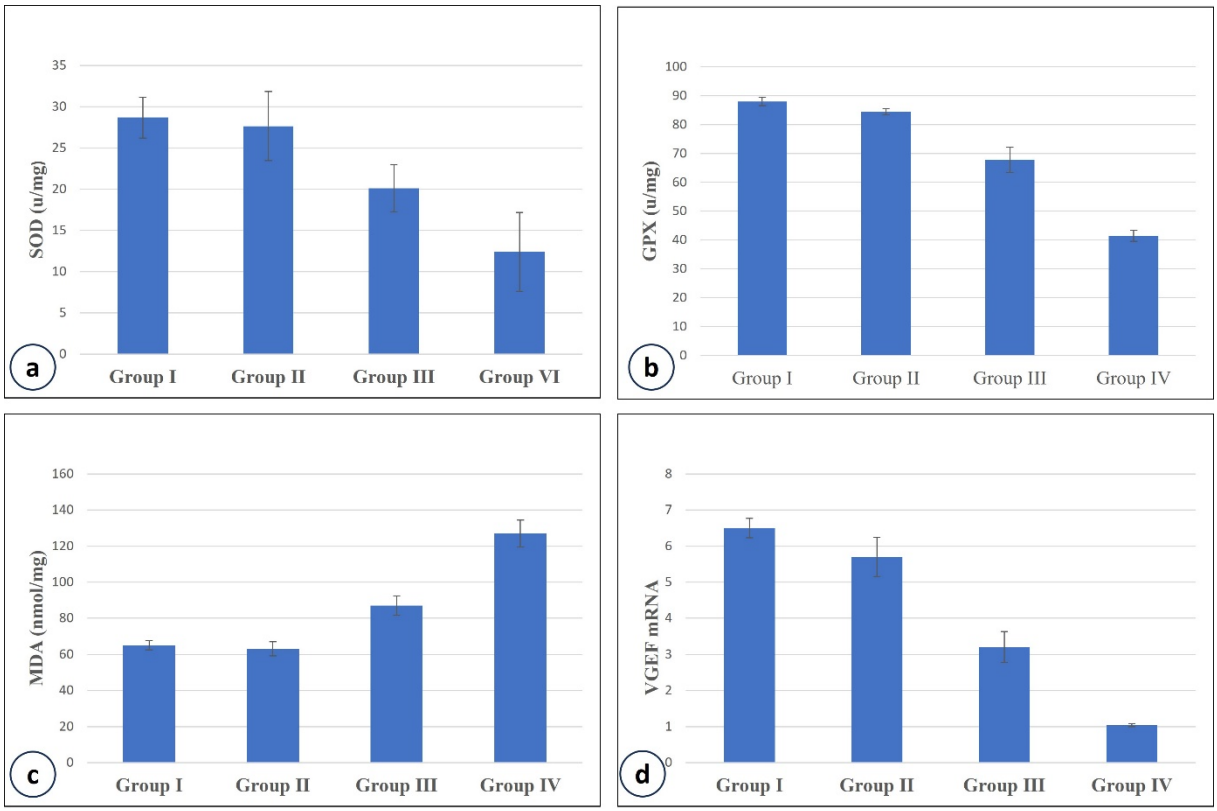

Figure 6. The mean (error bars: ± standard deviation); **A.** SOD level in the gastric homogenate in the studied groups; **B.** GPx level in the gastric homogenate in all groups; **C.** MDA level in the gastric homogenate in all groups; **D.** VEGF gene expression in the gastric homogenate in all groups. GPx — glutathione peroxidase; MDA — malondialdehyde; SOD — superoxide dismutase; VEGF — vascular endothelial growth factor.