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

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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 per group (prepubertal, adolescent, adult, and senile). 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, an increase in collagen content, and a decrease in mucous content with the advance of age. These morphological changes were associated with a significant decrease in both SOD and GPx activity, and an 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.* (Folia Morphol 2025; 84, 1: 127–139)

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

INTRODUCTION

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

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

Ageing gastropathy is an important and clinically relevant issue for an ageing world population due to prolonged lifespans. Older patients have a 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, ageing of the stomach is associated with an increased incidence of gastric cancer, which ranks as 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 ageing process. Within the cell, they can distort the structure of molecules, abolish their function, and create a molecular imbalance which can be observed at biomolecular level [5].

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

MATERIALS AND METHODS

Animals

This 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 standardised 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 anaesthetised by a mild ether inhalation and euthanised by cervical dislocation, after which specimens of the glandular stomach (corpus or pars glandularis) were subjected to 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, Cairo, Egypt, Catalogue 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, Carpinteria, CA, USA at a dilution 1/200). For detection of oxidative stress.

Histomorphometric study

Five non-overlapping fields from five sections (from each rat) of 20 rats per group were randomly chosen. The measurements were obtained using a Leica Qwin 500 C image analyser (Leica Imaging System Ltd, Cambridge, UK). 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 calculated for each slide,
2. Area percentage of collagen fibres 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 percentage of caspase-3 immuno-expression in gastric tissue at a magnification 400×,
5. Area percentage of iNOS immuno-expression in gastric tissue at a magnification 400×.

Area percentage of collagen fibres, optical density of glycogen, area percentage of caspase-3, and iNOS immuno-expression were done by transforming coloured images into grey images then masking the

positive areas by a red binary colour using image analyser software.

Biochemical study

ELISA: Gastric tissue homogenate was prepared for assessment of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) by an ELISA kit as described in the manufacturer's instructions (Biospes, Chongqing, China) and lipid peroxidation marker malondialdehyde (MDA) by an ELISA kit as described in the manufacturer's instructions (Biodiagnostics, Upton upon Severn, UK).

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 homogenisation in a TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentrations and purity were measured with an ultraviolet spectrophotometer [33].

Complementary DNA (cDNA) synthesis: cDNA was synthesised 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 10 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 minutes at 50°C, 10 minutes at 95°C and 40 cycles of denaturation for 15 seconds and annealing/extension at 60°C for 10 minutes. Data from real-time assays was calculated using v1.7 Sequence Detection Software (PE Biosystems, Foster City, CA, USA). Relative expression of studied gene mRNA was calculated using the comparative Ct method. All values were normalised 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 was analysed 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. A Tukey (post hoc) test was used for multiple comparisons between pairs of groups. Significance was accepted at $p \leq 0.05$.

RESULTS

Light microscopic results

Groups 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 recognised by their deep basophilic cytoplasm and basally situated rounded nuclei (Fig. 1C). Masson's trichrome-stained sections revealed a few thin collagen fibres 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 leukocytic 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. A few chief cells exhibited patchy destruction of cytoplasm, shrunken nuclei, and karyolysis (Fig. 2C). Masson's trichrome-stained sections showed a moderate increase in the amount of collagen fibres in the

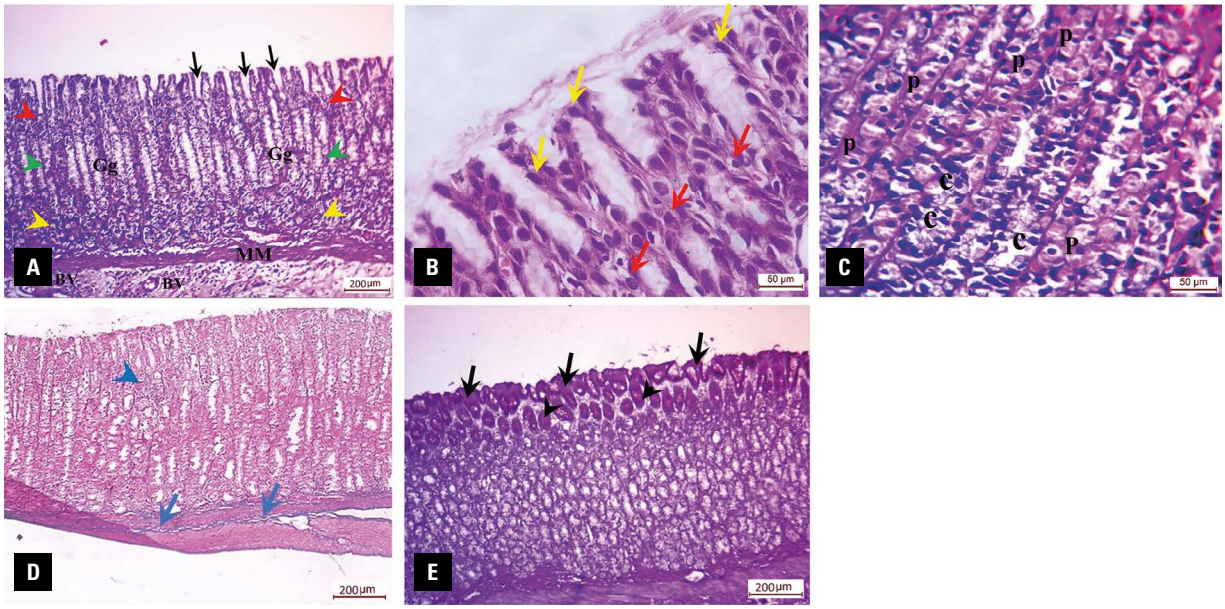

Figure 1. Photomicrographs of sections of gastric mucosa of rats from group I (prepubertal group): **A.** Surface epithelium is invaginated by gastric pits (arrows). Gg reach down to MM. Isthmus (red arrowheads), neck (green arrowheads) and base (yellow arrowheads) of glands are shown. Submucosa shows BV (H&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) (H&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 (H&E × 400); **D.** A few collagen fibres between gastric glands (arrowhead) and in submucosa (arrows) are illustrated (Masson's trichrome ×100); **E.** Strong positive PAS reaction is shown in surface epithelium (arrows) and in mucus neck cells (arrowheads) (PAS ×100). BV — blood vessels; Gg — gastric glands; H&E — haematoxylin and eosin; MM — muscularis mucosa.

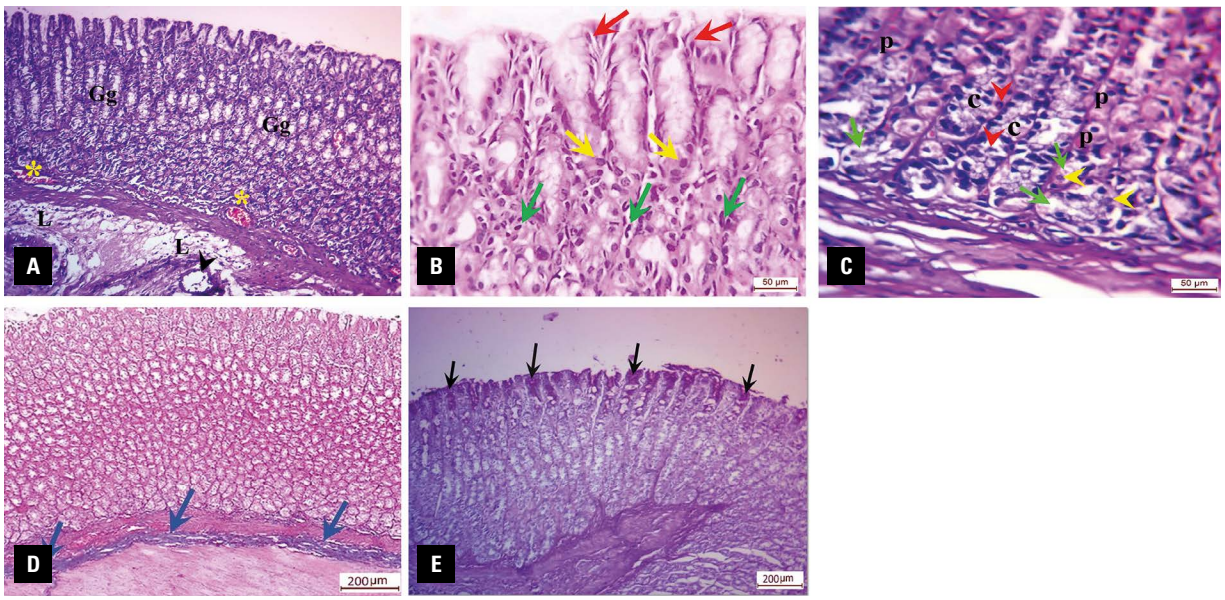

Figure 2. Photomicrographs of sections of gastric mucosa of rats from group III (adult): **A.** Apparently normal Gg are illustrated. Note blood vessels (*) at basal part of glands. Submucosa shows mild leukocytic infiltration (L). Blood vessel in submucosa (arrowhead) is demonstrated (H&E × 100); **B.** Surface mucous cells (red arrows) and mucous neck cells (yellow arrows) are apparently normal except for a few pyknotic nuclei (green arrows) (H&E × 400); **C.** Apparently normal parietal cells (P) and chief cells (C) are demonstrated. A few chief cells exhibit patchy destruction of cytoplasm (green arrows), shrunken nuclei (red arrowheads), and karyolysis (yellow arrowheads) (H&E × 400); **D.** Moderate amount of collagen fibres are seen in submucosa (arrows) (Masson's trichrome ×100); **E.** Surface epithelium exhibits moderate PAS reaction (arrows) (PAS ×100). Gg — gastric glands; H&E — haematoxylin and eosin; PAS — periodic acid Schiff.

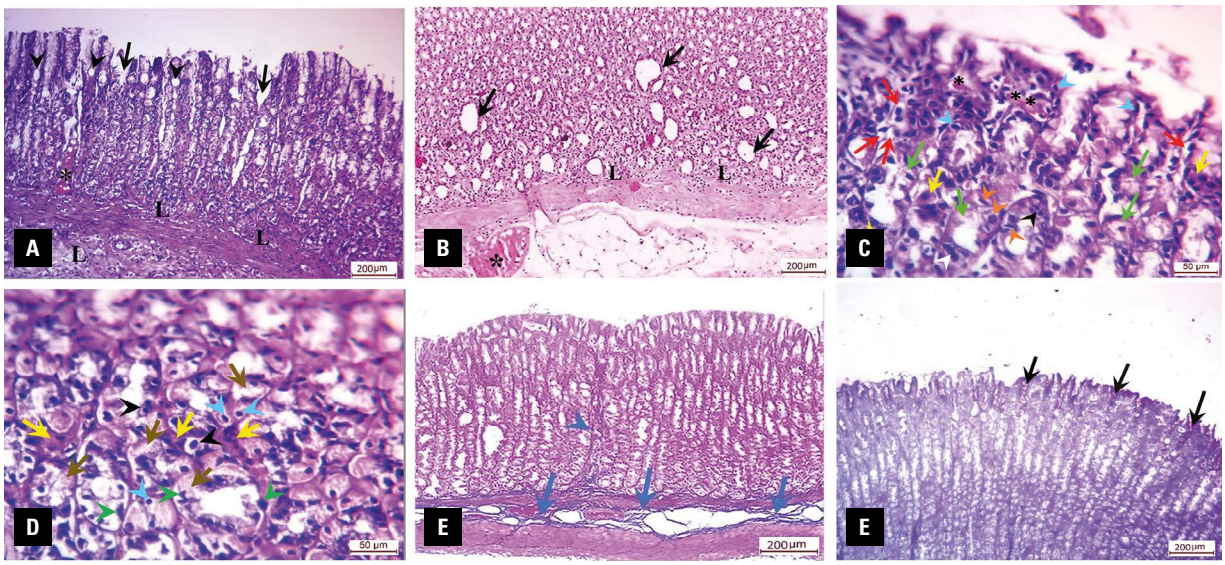

Figure 3. Photomicrographs of sections of gastric mucosa of rats from group IV (senile): **A.** Erosions of gastric mucosa (arrows) and cytoplasmic vacuolations (arrowheads) are illustrated in isthmus region of glands. Leukocytic infiltration (L) is demonstrated at bases of glands and in submucosa. Note extravasation of blood (*) between gastric glands (H&E × 100); **B.** Lumina of gastric glands are widened (arrows) with marked leukocytic infiltration (L) in basal part of glands. Blood vessel (*) is demonstrated in submucosa (H&E × 100); **C.** Mucous cells show 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 bizarrely-shaped nuclei (orange arrowheads) are shown. Note apoptotic-like cells (yellow arrows) and extravasation of blood (*) (H&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 (H&E×400); **E.** Moderate amount of collagen fibres between gastric glands (arrowhead) and abundant collagen fibres in submucosa (arrows) are shown (Masson's trichrome ×100); **F.** Weak PAS reaction is seen in surface epithelium (arrows) (PAS ×100). H&E — haematoxylin and eosin; PAS — periodic acid Schiff.

submucosa (Fig. 2D). Sections stained with PAS revealed a 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 bizarrely 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, bizarrely shaped nuclei and peri-nuclear vacuolation were seen (Fig. 3D). Masson's trichrome-stained sections showed an apparent increase in the amount of collagen fibres surrounding gastric glands and in the submucosa (Fig. 3E). Sections stained with PAS revealed weak reaction of the surface epithelium (Fig. 3F).

Immunohistochemical results

Groups I (prepubertal) and II (adolescent): 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).

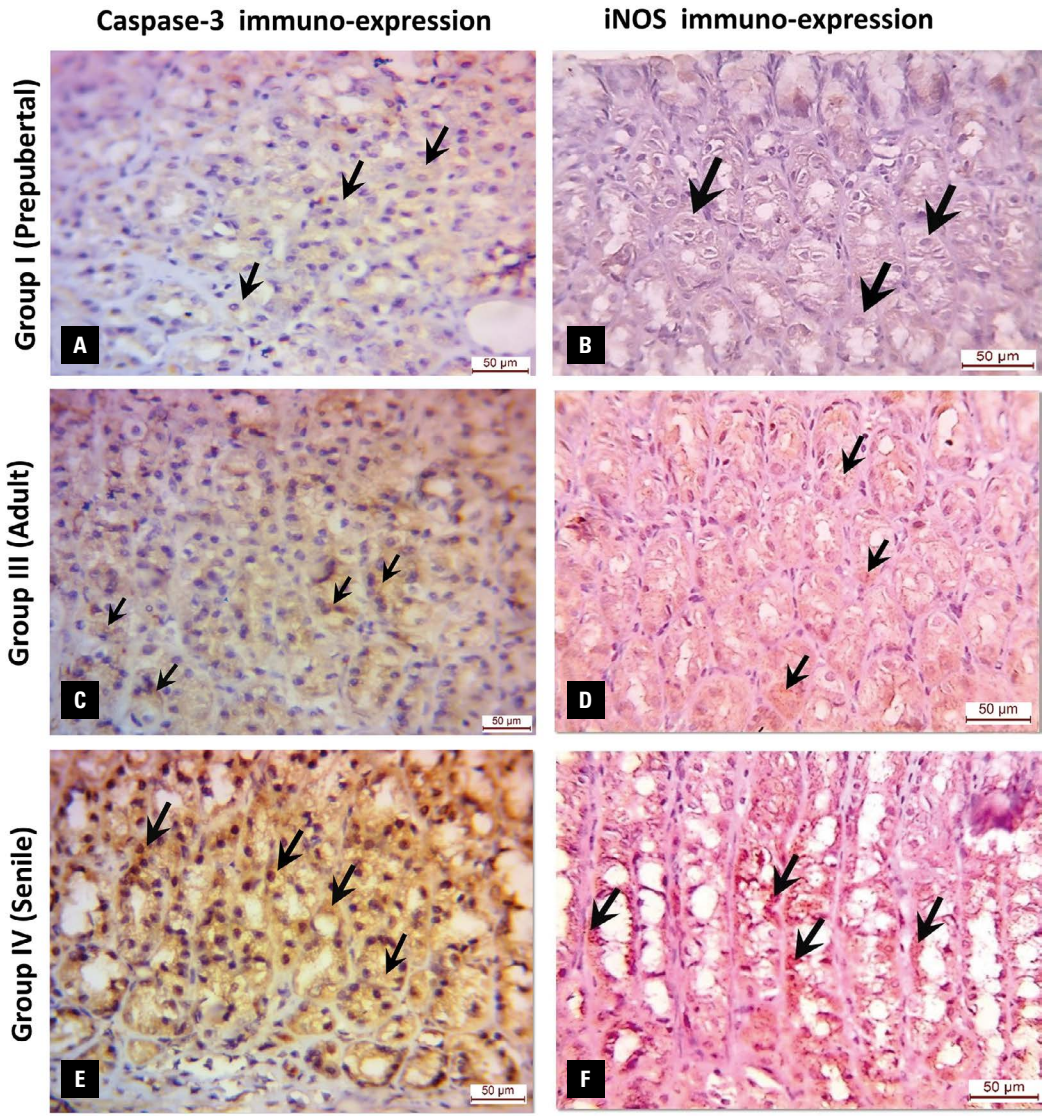

Figure 4. Photomicrograph of rat gastric mucosa: **A.** Group I (prepubertal group) shows weak caspase-3 immunoreaction in gastric glands (arrows) (Caspase-3×400); **B.** Section from group I (prepubertal group) demonstrates weak inducible nitric oxide synthase (iNOS) immunoreactivity in gastric glands (arrows) (iNOS×400); **C.** In group III (adult group), moderate caspase-3 immunoreaction (arrows) is shown in gastric glands (Caspase-3 × 400); **D.** Group III (adult group) reveals moderate iNOS immunoreaction in gastric glands (arrows) (iNOS × 400); **E.** Group IV (senile group) exhibits moderate caspase-3 immunoreaction in gastric glands (arrows) (Caspase-3 × 400); **F.** Group IV (senile group) reveals strong iNOS immunoreactivity in gastric glands (arrows) (iNOS × 400).

Histomorphometric results

Thickness of gastric mucosa in H&E-stained sections: There was a statistically significant age-related decrease in gastric mucosal thickness in senile rats compared to prepubertal, adolescent, and adult rats ($p < 0.001$* in the three groups). Gastric mucosal thickness decreased in adolescent rats 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 compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*) (Tab. 1, Fig. 5A).

Area percentage of collagen fibres in Masson's trichrome-stained sections: The collagen area percentage in the peri-glandular and submucosal regions increased in senile rats compared to prepubertal, adolescent and adult rats. This increase was found to be statistically significant when compared to prepubertal ($p < 0.001$*) and adolescent rats ($p < 0.001$*), although it was non-significant when compared to adult rats ($p = 0.209$). In adolescent rats, there was a statistically non-significant increase in collagen area percentage compared to prepubertal rats ($p = 0.757$). Regarding adult rats, there was a significant increase

Table 1. Mean values of gastric mucosal thickness, area percentage of collagen fibres, optical density of mucous content, area percentage of caspase-3, and iNOS immuno-expression in all rat groups.

Groups	Gastric mucosal thickness (μm) (mean ± SD)	Area percentage of collagen fibres (mean ± SD)	Optical density of mucous content (mean ± SD)	Area percentage of caspase-3 immuno--expression (mean ± SD)	Area percentage of iNOS immuno--expression (mean ± SD)
I (Prepubertal)	525 ± 95.6	0.60 ± 0.54	65.04 ± 1.5	0.898 ± 0.213	0.87 ± 0.329
II (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**

*Significant ≤ 0.05 vs. groups I and II; **Significant ≤ 0.05 vs. groups I, II, and III; ***Significant ≤ 0.05 vs. groups I and II.

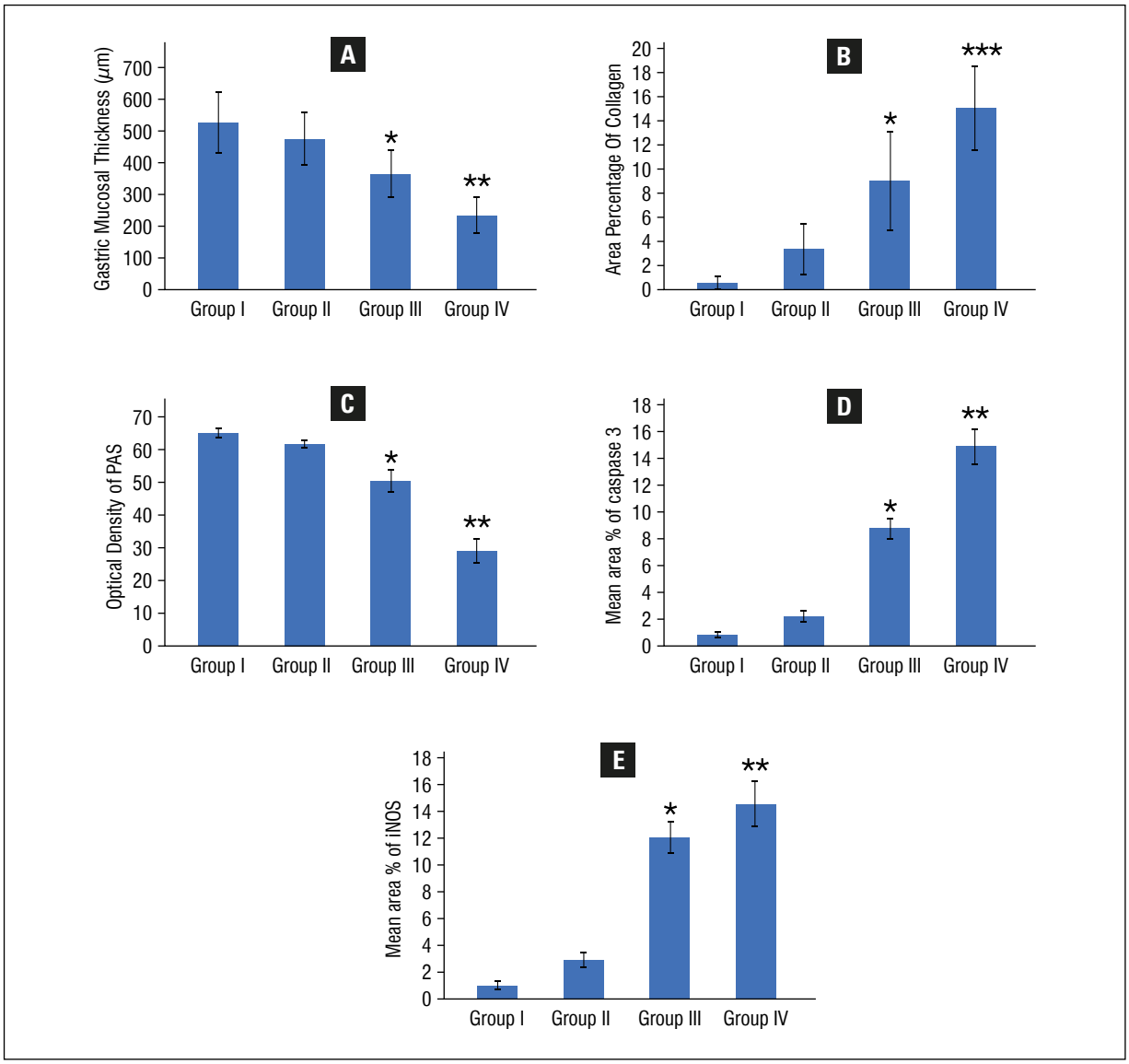

Figure 5. Mean (error bars: ± standard deviation); **A.** Gastric mucosal thickness (μm) H&E stained sections in studied groups; **B.** Area percentage of collagen in Masson's trichrome-stained sections in studied groups; **C.** Optical density of PAS-stained sections in studied groups; **D.** Area percentage of caspase-3 immunoexpressioin in studied groups; **E.** Area percentage of iNOS immunoexpressioin in studied groups. *Significant ≤ 0.05 vs. groups I and II; **Significant ≤ 0.05 vs. groups I, II, and III; ***Significant ≤ 0.05 vs. groups I and II. H&E — haematoxylin and eosin; iNOS — inducible nitric oxide synthase; PAS — periodic acid Schiff.

in collagen area percentage compared to adolescent and prepubertal rats (p = 0.032* and p = 0.001* respectively) (Tab. 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 compared to prepubertal, adolescent and adult rats (p < 0.001* in the three groups). A non-significant decrease in the mucous content of gastric glands in adolescent rats was found compared to prepubertal rats (p = = 1.000). In adult rats, there was a significant decrease in mucous secretion compared to adolescent and prepubertal rats (p = 0.011* and p = 0.001* respectively) (Tab. 1, Fig. 5C).

Area percentage of caspase-3 immunoexpression in gastric tissue: There was a statistically significant increase in the area percentage of caspase-3 immunoexpression in the gastric homogenates of senile rats compared to prepubertal, adolescent and adult rats (p < 0.001* in the three groups). In adolescent rats, there was a statistically non-significant increase in area percentage 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*) (Tab. 1, Fig. 5D).

Area percentage of iNOS immuno-expression in 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). A non-significant increase in iNOS immuno-expression in adolescent rats was found compared to prepubertal rats (p = 0.070). In adult rats, there was a significant increase in iNOS immuno-expression compared to adolescent (p < 0.001*) and prepubertal rats (p < 0.001*) (Tab. 1, Fig. 5E).

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*). 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 compared to adolescent (p = 0.034*) and prepubertal rats (p = = 0.013*) (Tab. 2, Fig. 6A).

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

Malondialdehyde: Senile rats showed a statistically significant increase in the mean MDA level in the gastric tissue compared 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 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) (Tab. 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 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 compared to prepubertal rats (p = 0.062*) (Tab. 2, Fig. 6D).

Table 2. Mean values of SOD, GPx, MDA and *VEGF* gene expression in 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**

*Significant ≤ 0.05 vs. groups I and II; **Significant ≤ 0.05 vs. groups I, II, and III. GPx — glutathione peroxidase; MDA — malondialdehyde; SOD — superoxide dismutase; VEGF — vascular endothelial growth factor.

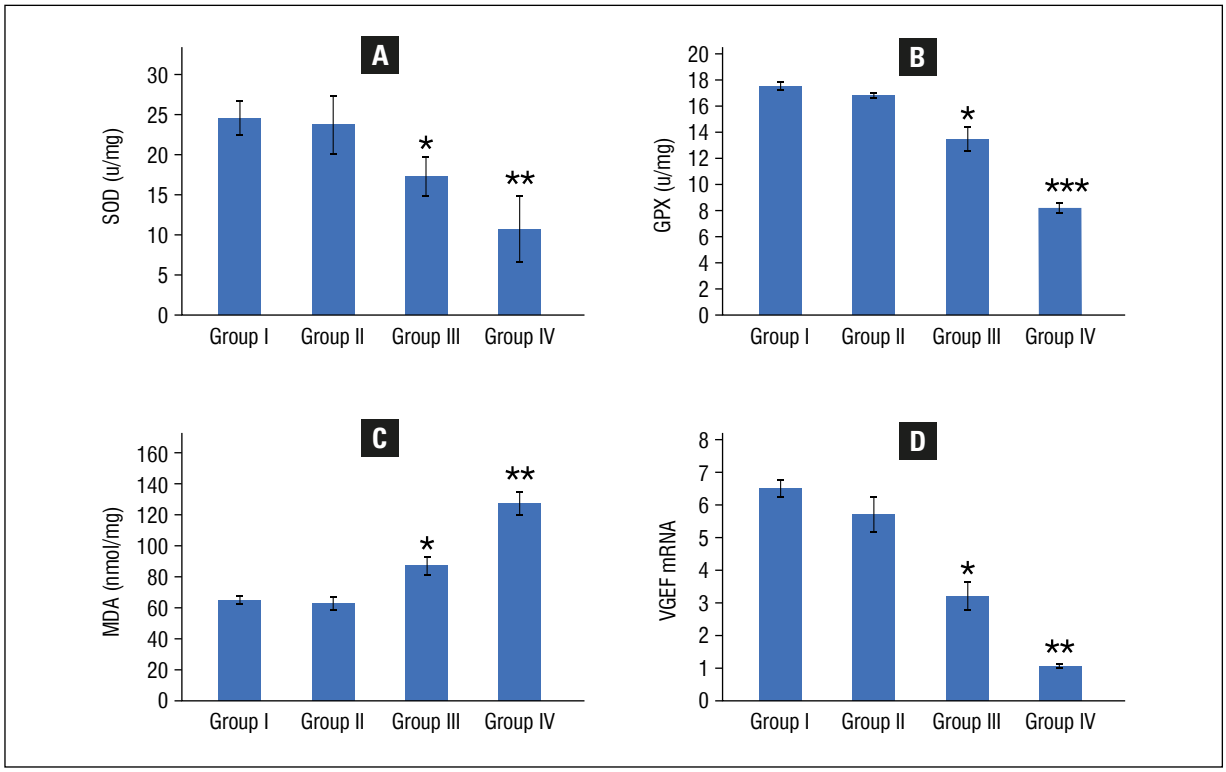

Figure 6. Mean (error bars: ± standard deviation); **A.** SOD level in gastric homogenate in studied groups; **B.** GPx level in gastric homogenate in all groups; **C.** MDA level in gastric homogenate in all groups; **D.** *VEGF* gene expression in gastric homogenate in all groups. *Significant ≤ 0.05 vs. groups I and II; **Significant ≤ 0.05 vs. groups I, II, and III. GPx — glutathione peroxidase; MDA — malondialdehyde; SOD — superoxide dismutase; VEGF — vascular endothelial growth factor.

DISCUSSION

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

We found histological examination of gastric mucosa of prepubertal and adolescent rats to show normal histology. There was a non-significant decrease in gastric mucosal thickness and mucous content, in addition to a non-significant increase in collagen in the gastric mucosa of adolescent rats compared to prepubertal rats. In adult rats, despite mild morphological changes, there was a statistically significant increase in collagen fibres, as well as a decrease in mucus content and gastric mucosal thickness in this group compared to prepubertal and adolescent rats. Contradicting this, El-Shall [12] reported a strong positive PAS reaction in surface and mucous neck cells in 18 month-old rats, indicating unaffected mucus secretion in this age group. The differences in findings El-Shall's work and our 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 our present study showed few cells with pyknotic nuclei and degenerated cytoplasm. This finding was associated with moderate caspase 3 immunoexpression, which was confirmed by the statistically significant increase in caspase-3 expression 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 our present study, as there was a significant increase in iNOS immunoexpression in the gastric mucosa of this group compared to the 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 young ones. Similarly, Vucevic et al. [31] found that the MDA level in the gastric tissue of 18-month-old rats was significantly higher than in 3-month-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 ageing process.

Examination of gastric tissue from the senile group in our present 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 are consistent with the data provided by Lutnicki et al. [21], who reported lack of organisation 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 et al. [18] demonstrated that microscopic features of aged stomachs were as normal as young ones except for increased vascularisation and congestion, especially at the bottom of the glands.

The fibrosis of gastric mucosa of the senile rats observed in our present study was confirmed by the significant increase in collagen area percentages in the peri-glandular and submucosal regions compared to the 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 fibres increased significantly with advancing age. However, the significant increase in collagen area percentage of gastric tissue found in our present 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 the gastric glands accompanied by a significant increase in connective tissue replacing glandular cells in ageing rats. On the other hand, Brito et al. [8] reported finding no alterations of collagen fibres in the gastric mucosa of old rats.

The differences in the results between these authors and ourselves in our present study might be due to a different classification of age groups, as these authors considered 18-month-old rats as being in the senile group, while we considered them to be adults. 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 et al. [18] reported an obvious decrease in the thickness of gastric mucosa in aged rats compared to young ones. They pointed out that insufficient secretion of prostaglandins and bicarbonate results in a decrease in the thickness of the mucus layer that is naturally associated with a decreased number of glandular cells including glandular mucus.

Contradicting our present findings, Majumdar et al. [23] reported an increase in overall thickness of the gastric mucosa in 24-month-old rats compared to four-month-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 our present study, which is consistent with the work carried out by El-Shall [12]. We attributed the increased incidence of fundic ulcers observed in the senile group to increased susceptibility of the mucosa to various damaging agents, together with impediments to the repair process. On the other hand, no macroscopic ulcers were detected in the stomachs of rats of any age by Marmol et al. [24].

In our 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 agree with El-Shall [12], who reported a decrease in PAS reaction in mucous neck cells in 24-month-old rats. El-Shall attributed that to decreased secretory activity of the gastric mucosal cells, with a decreased number of intracytoplasmic secretory granules at the ultrastructural level.

In the recent study by Kirmizikan et al. [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 a relative loss of glandular cells with subsequent thinning of the surface mucus layer indicating a poor mucosal barrier in aged animals.

In our present work, inflammation of gastric mucosa with age was observed in the adult and senile groups as evidenced by leukocytic 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 our present work, histological examination of gastric specimens obtained from senile rats showed apoptotic-like cells with deeply acidophilic cytoplasm and condensed nuclear chromatin. This was confirmed by a significant increase in caspase-3 immunoexpression in the gastric tissue of senile rats compared to prepubertal, adolescent and adult rats. This data agreed with Tarnawski et al. [30], who found a significant

increase in proapoptotic cleaved caspase-3 and a reduction of antiapoptotic surviving in the gastric mucosa of aged rats. They clarified that the imbalance between proapoptotic and antiapoptotic factors results in increased apoptosis and partial gastric mucosal atrophy in ageing rats, which also provides an explanation for its increased susceptibility to injury.

In alignment with our present study, increased caspase-3 activity in very old rats has 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 our present study, there was a significant age-related increase in the immunoexpression of iNOS in the gastric mucosa of senile rats 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 stomachs 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 stomachs of 24-month-old rats.

It has been reported that the balance between the generation of oxygen free radicals and the activity of the antioxidative system can play a significant role in the ageing 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 neutralise 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 our present study, an age-related significant decrease in VEGF expression was observed in the gastric tissues of the adult and senile groups compared to young ones. This accords 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 ageing rats.

It has been reported that hypoxia is a potent stimulus for *VEGF* gene activation [16]; therefore, it could be expected that ageing gastric mucosa might have increased VEGF expression. However, Ahluwalia et al. [1] demonstrated that ageing gastric mucosa did not express higher VEGF levels, despite increased tissue hypoxia, indicating that the 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 ageing 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 our present work.

Age-related deficiency in mucosal repair secondary to reduced VEGF expression in the stomachs of senile rats was also reported by Majumdar [23] and Kang et al. [17]. The latter authors reported that VEGF expression was significantly decreased in 18-month-old and two-year-old rats compared to 7-month-old rats.

CONCLUSIONS AND RECOMMENDATIONS

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

ARTICLE INFORMATION AND DECLARATIONS

Data availability statement

Data is available upon reasonable request from the corresponding author.

Ethics statement

This 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

SAFM: conceptualisation; AAERA, MMN: supervision and guiding authors; DMS: writing manuscript

and data analysis; MME: performing experiment and statistical analysis.

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Conflict of interest

The authors declared no conflict of interest.

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