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Original article

Ageing-Related Alterations in Rat Stomach and Intestines: A Microscopic Approach

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Abstract

Objective: Controversial results related with the ageing-related histological and stereological changes have been reported. We aimed to investigate the effects of ageing on stomach and jejunum of Sprague-Dawley rats at light microscopic level. ***Methods:*** In this study, 14 male Sprague-Dawley rats were used. The rats were divided into two groups as young and aged. The ageing was created via pinealectomy. Stomach and jejunum samples of each rat were evaluated by light microscope. ***Results:*** There were no statistically significant difference among groups regarding the gastric luminal mucus thickness and the numbers of goblet cell within the crypts ($p>0,05$). In comparison with the aged group the gastric mucosal thickness, the number PCNA positive cells at the gastric surface epithelium and the epithelium of villi, the depth of crypt were significantly decreased in young group ($p<0.05$). The mean number TUNEL positive cells at the surface epithelium of stomach and the epithelium of villi, the length of the intestinal villi, the numbers of goblet cell within the epithelium of the villi were significantly increased in the aged group compared to the young group ($p<0.05$). ***Conclusion:*** It was concluded that there are histological changes in the ageing small intestine and stomach.

Keywords: *Ageing, histology, small intestine, stomach*

Introduction

Ageing is biological process that causes loss of tissue and organ function. DNA damage accumulation, telomere shortening, high amount of reactive oxygen species production, abnormal gene activities, metabolic changes, mitochondrial dysfunction are among the changes detected in ageing cells [1]. Many theories have been proposed for the ageing mechanism so far of which the mitochondrial ageing theory has come into prominence in recent years. According to that theory, reactive oxygen species (ROS) produced mainly in the inner mitochondrial membrane are an undesirable undetermined byproduct of aerobic metabolism targeting various cellular structures including membrane proteins and lipids and DNA. The accumulation of low to moderate levels of ROS is generally counterbalanced by the cell's endogenous antioxidant defense system. If the amount of ROS increases, and if these products destroy the apparatus by which antioxidant agents are produced, the cellular defense system is eventually incapacitated. It appears that higher levels of ROS induce necrotic cell death whereas lower levels lead to apoptosis [2]. Oxidative stress plays a role in the pathogenesis of various diseases including neurodegenerative diseases and cancer as well as ageing itself and ageing-related organ damage. The main prediction of the mitochondrial theory of ageing is that the mitochondria drive the cell oxidative stress and thus causes senescence [3,4].

Although ageing-related noticeable changes are seen in the skin and skin appendages, in fact all internal organs are deeply affected. Stomach and intestine are among these organs undergoing physiological and pathological alterations in the course of ageing. Clinical and experimental studies have reported ageing-related changes including decrease in bicarbonate secretion, gastric blood flow rate, delay in gastric emptying and mucosal changes related with gastric morphology and function [5-7]. Various age-dependent changes including disruption of epithelial tight-junctions, reduction in micro-vessel volume and absorption, and changes in the microbiota have been reported related with intestinal morphology and function [8,9].

At the present study we tried to investigate age-related histopathological changes and cellular proliferation and apoptosis status on stomach and small intestines of Sprague-Dawley rats.

Materials and methods

Experimental protocol: Fourteen male 4-weeks-old Sprague-Dawley rats weighing 150–200g were placed in a constant temperature (21±2°C) and humidity (60±5%) controlled room in which a 12:12h light dark cycle was maintained. They were allowed free access to a commercial standard died and water ad libitum. Animals placed in cages two by two were divided into two groups: Group I (n=7) and group II (n=7) were designated as sham-pinealectomized and pinealectomized (Px) rat groups; respectively. Pinealectomy was performed to half of the rats under general anesthesia of ketamine/ksilazin (30/15 mg/kg) according to the method of Kuzsak &Rodin [10]. Pinealectomy was confirmed by the histological evaluation of the gland. Pinealectomized animals were considered as aged animals 6 months after the procedure.

All experimental procedures were reviewed and approved by the Animal Experimental Committee of Bezmialem Vakıf University and performed according to Bezmialem Vakıf University Animal Research Center guidelines on the care and use of animals.

Histopathological analysis: Following decapitation, stomach, and small intestines (jejenum) were removed, and samples were fixed in 10% buffered neutral formalin and dehydrated through ascending alcohols, cleared with xylene and embedded in paraffin blocks. 5 µm

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thick sections were taken from paraffin blocks using LEICA RM2245 microtome, mounted on slides. Paraffin slides stained with Hematoxylin and Eosin (H&E), and Periodic Acid Schiff (PAS) staining techniques were examined by a blind observer using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1c camera and the Nikon NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc., Tokyo, Japan).

Histological processes

After decapitation, stomach and intestine tissues were fixed 48 h in the 10% formalin, dehydrated through ascending alcohols, cleared with xylene and embedded in paraffin blocks. 5 µm thick sections were taken from paraffin blocks using LEICA RM2245 microtome, mounted on slides. Gastric mucosal thickness and luminal mucus thickness were measured in cross sections of 20 area per sample. Intestinal villus and crypt length were measured in cross sections of 20 villi/crypt per sample. The number of goblet cell in villus epithelium and glandular epithelium were calculated in cross sections of 20 villi/crypt per sample.

Immunohistochemistry

Anti-PCNA (Polyclonal Antibody) immunohistochemistry (ThermoFisher Scientific, 991143) in order to get clues about for proliferation rate and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling- (TUNEL) method (Merck Millipore, S7100) in order to get clues for apoptosis rate were used. Sections were kept in an oven at 37 0C overnight, then deparaffinized with xylene and dehydrated in decreasing grades of alcohol solutions. The immunohistochemistry protocols were applied to the sections according to the instructions of the manufacturer. Sections were counter-stained with Mayer's hematoxylin. PCNA positive cells and TUNEL positive cells were counted in 10 area of gastric and intestinal villi epithelium under 40X magnification. All samples were examined by blind observe using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1c camera, and Nikon NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc; Tokyo, Japan).

Statistical analysis

All statistical analysis was performed using GraphPad Prism 8 (USA) program. Student's T-test was used for the comparison of the results between groups. P <0.05 was considered as significant.

Results

Changes in aged stomachs: Stomachs of young rats were healthy as expected (**Figure 1A**). Microscopical features of aged stomachs were also pretty normal except increased vascularization and congestion especially at the bottom of lamina propria and increased density of connective tissue of lamina propria. Additionally, the lamina muscularis mucosa seemed to be thickened. Nevertheless, the thickness of mucosa was obviously decreased (**Figure 1B**). Luminal mucus layer was also seemed to be decreased at PAS-stained sections of aged stomachs versus young ones. Additionally, loss of PAS positive stained glandular mucous neck cells was obvious at the sections of aged rats (**Figure 1C, 1D**). Mean gastric mucosal thickness of young rats was 274.965±23.599 µm whereas that of aged rats was 223.435±35.294 µm ($p<0.05$) (**Figure 1E, Table I**). Mean luminal mucus thickness of young rats was 6.939±1.345 µm whereas that of aged rats was 4.894±1.328 µm ($p>0.05$) (**Figure 1F, Table I**). The ratio of proliferation and apoptosis was also changed in the stomachs of aged rats versus young rats. PCNA positive staining was more obvious at the sections of young stomachs versus aged ones (**Figures 2A, 2B; respectively**) whereas TUNEL-positive staining was more obvious at the sections of stomachs of aged rats versus young ones (**Figures 2D, 2C; respectively**). The mean number PCNA positive cells at the surface epithelium was significantly decreased in the aged group compared to the young group ($p<0.05$) whereas the mean number of TUNEL positive cells at the surface epithelium was significantly increased in the aged group compared to the young group ($p<0.05$) (**Table I, Figures 2E, 2F; respectively**).

Changes in aged intestines: Hematoxylin and eosin-stained jejunum sections of young rats were healthy as expected (**Figure 3A**). The microscopical features of those of aged rats also seemed normal except the changes in the length of the villi and depth of the glands (intestinal crypts) (**Figures 3A, 3B**). Mean length of the intestinal villi of aged rats was higher than that of the young rats (234.738±38.804 µm vs 127.234±13.427 µm; $p<0.05$) whereas mean depth of glands of aged rats was lower than that of the young rats (71.676±17.972 µm vs 277.664±23.874 µm; $p<0.05$) (**Figures 3A-3D, Table II**). PAS-stained sections revealed the changes in the distribution of mucus producing goblet cells. Goblet cell number was seemed to be increased in aged rats (**Figures 4A,B**). Mean numbers of goblet cell within the epithelium of the villi ($p<0.05$) and that of the crypts were decreased in aged rats compared to the young rats (**Figures 4C, 4D, Table II**). The mean number of PCNA positive cells in the epithelium of villi were decreased in the aged group compared to the young group ($p<0.05$) (**Figures 5A, 5B, Table II**) while mean number of TUNEL positive cells in the epithelium of villi were increased in the elderly group compared to the young group ($p<0.05$) (**Figures 5C, 5D, Table II**).

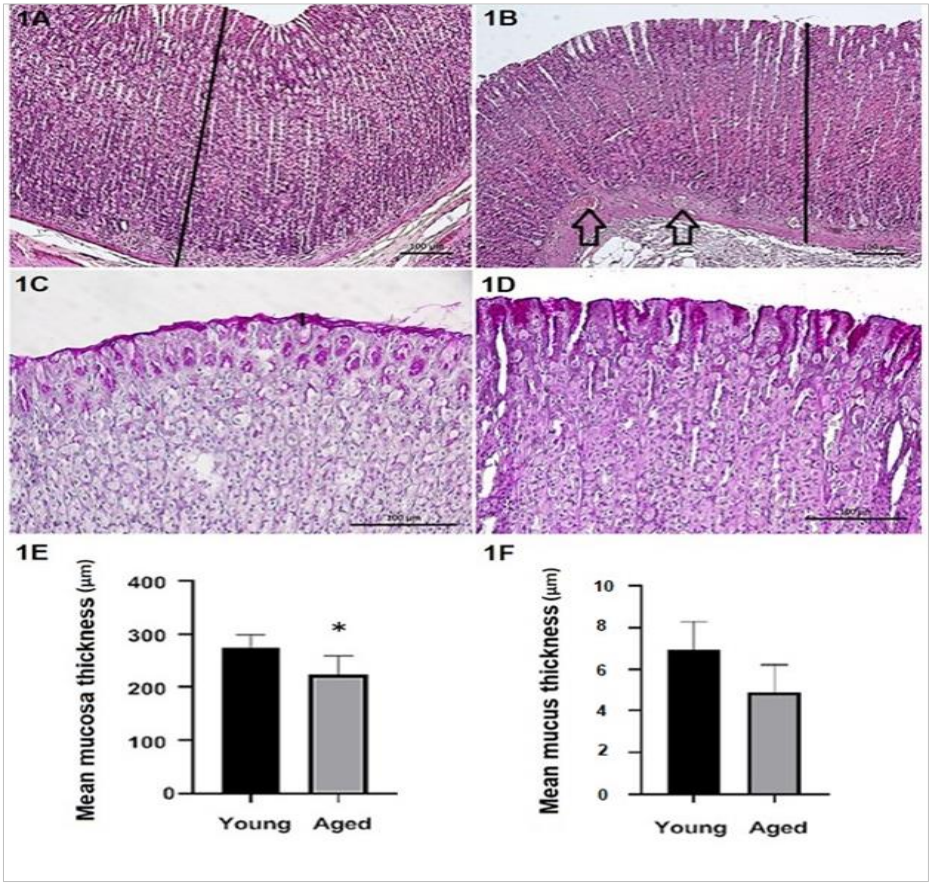

Figure 1: Hematoxylin and eosin- and PAS-stained stomach sections of young (1A, 1C; respectively) and aged (1B, 1D; respectively) rats. Ageing-related histological and stereological changes in the rat stomach. Decrease in the thickness of mucosa (black lines) (1A, vs 1B), and luminal mucus and PAS positive stained glandular mucous neck cell loss (1C vs 1D) are obvious. Vascularization and congestion (arrows) are observed (1B). Mean thickness of mucosa ($p<0.05$) and luminal mucus layer of aged rats are less than those of young rats (1E, 1F). Hematoxylin and eosin staining technique (A, B), PAS staining technique (C, D).

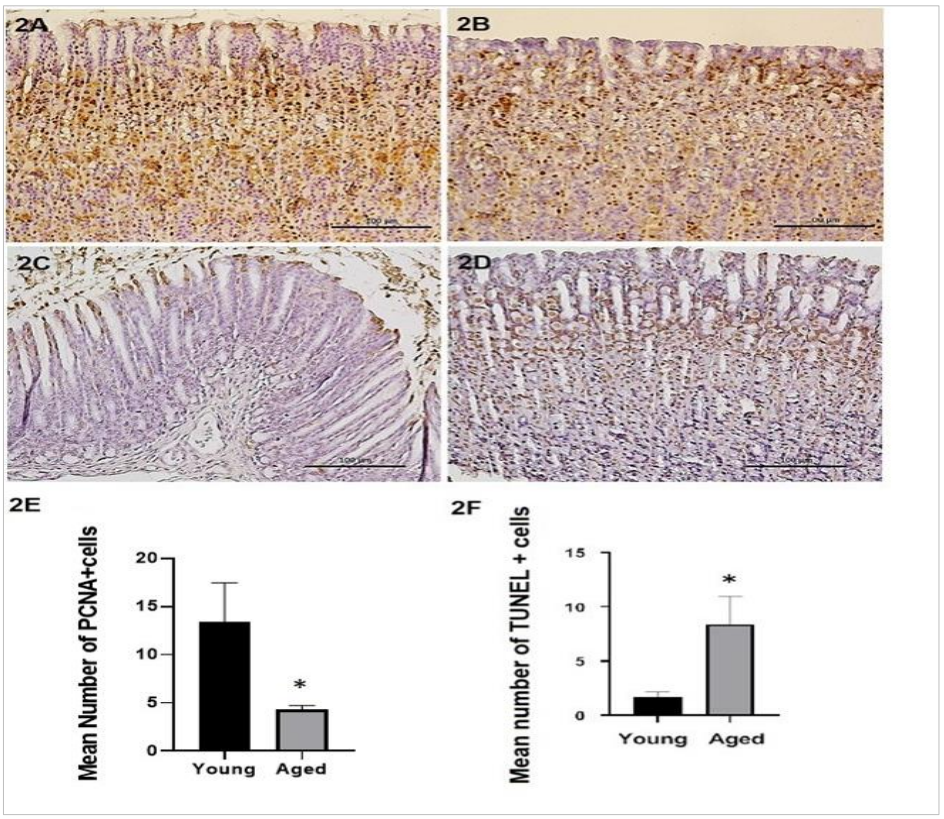

Figure 2. Proliferation and apoptosis status of the surface epithelium of the stomachs of young rats (2A, 2C; respectively) and those of the aged rats (2B, 2D; respectively). PCNA positive staining is prominent in young stomachs whereas TUNEL positive staining is prominent in aged rats. Mean number of PCNA positive cells is significantly decreased in the aged group vs young group ($p<0.05$) whereas mean number of TUNEL positive cells is increased in the aged group vs young group ($p<0.05$) (2E, 2F).

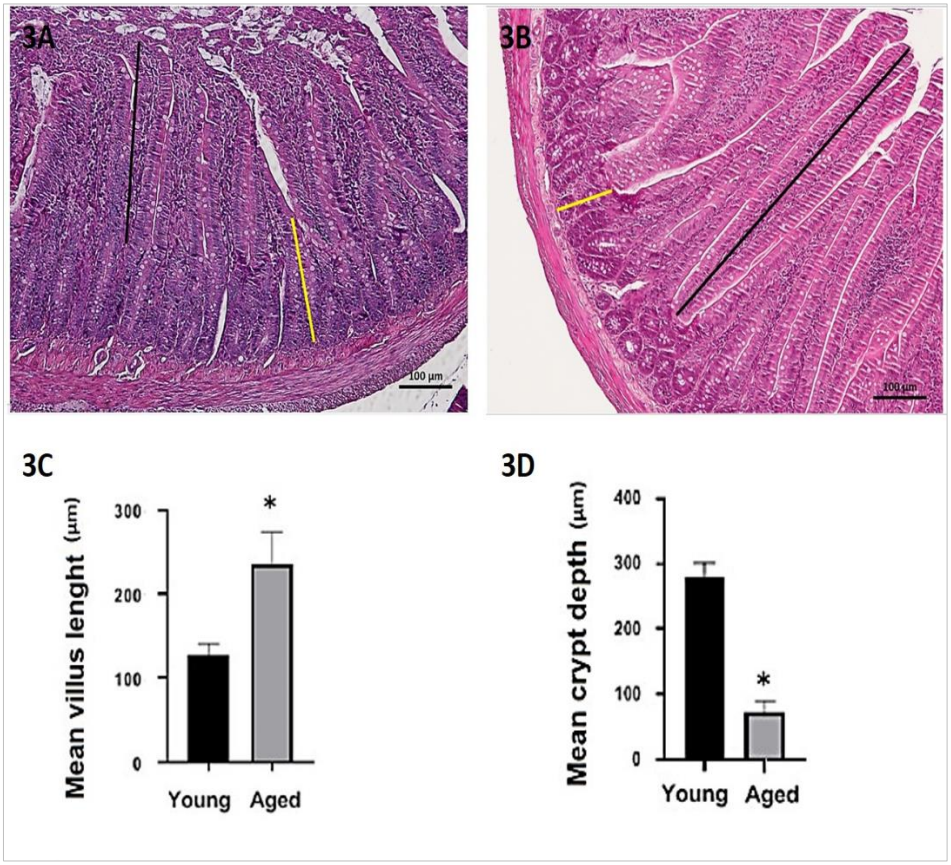

Figure 3: Hematoxylin and eosin-stained jejenum sections of young (3A) and aged (3B) rats. The cellular features of young and aged intestines seem healthy. The length of the villi (black lines) and depth of the glands (intestinal crypts) (yellow lines) seems changed (A, B; respectively). Mean length of the intestinal villi of aged rats is higher than that of the young rats ($p<0.05$) whereas mean depth of glands of aged rats is lower than that of the young rats ($p<0.05$) (3C, D). Hematoxylin and eosin staining technique.

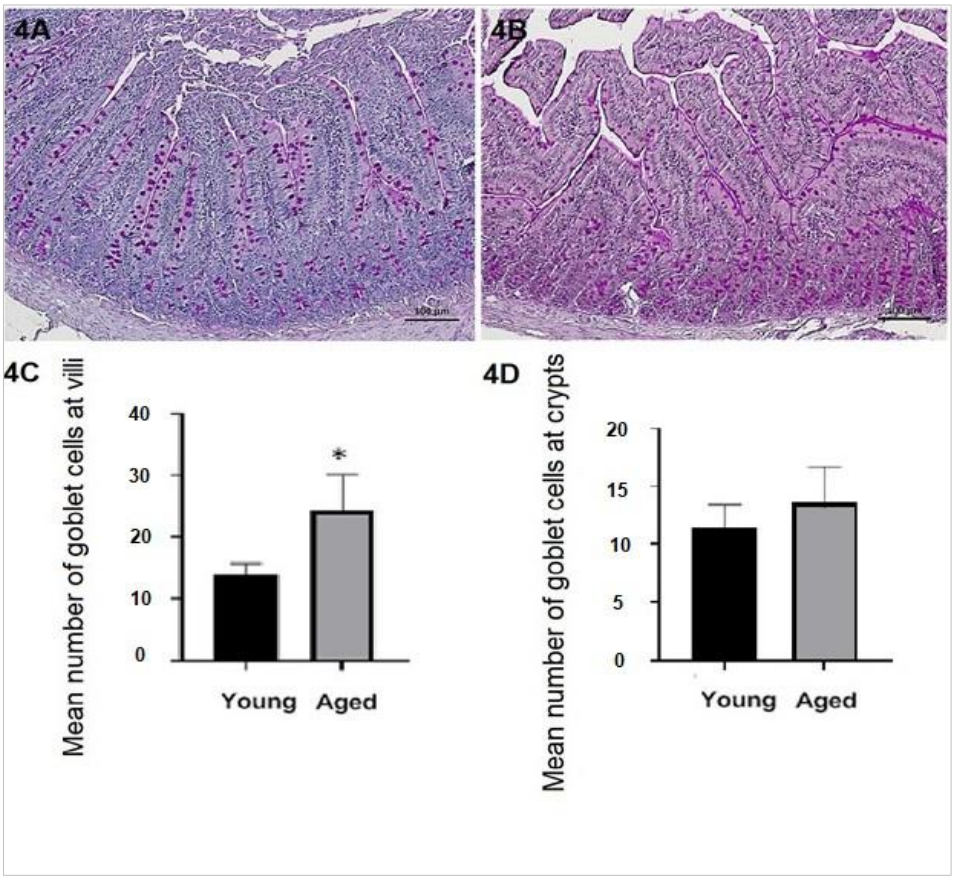

Figure 4: PAS-stained jejenum sections of young (4A) and aged (4B) rats. Goblet cell number was seemed to be increased in aged rats. Mean numbers of goblet cell within the epithelium of the villi ($p<0.05$) and that of the crypts are increased in aged rats compared to the young rats (4C, 4D).

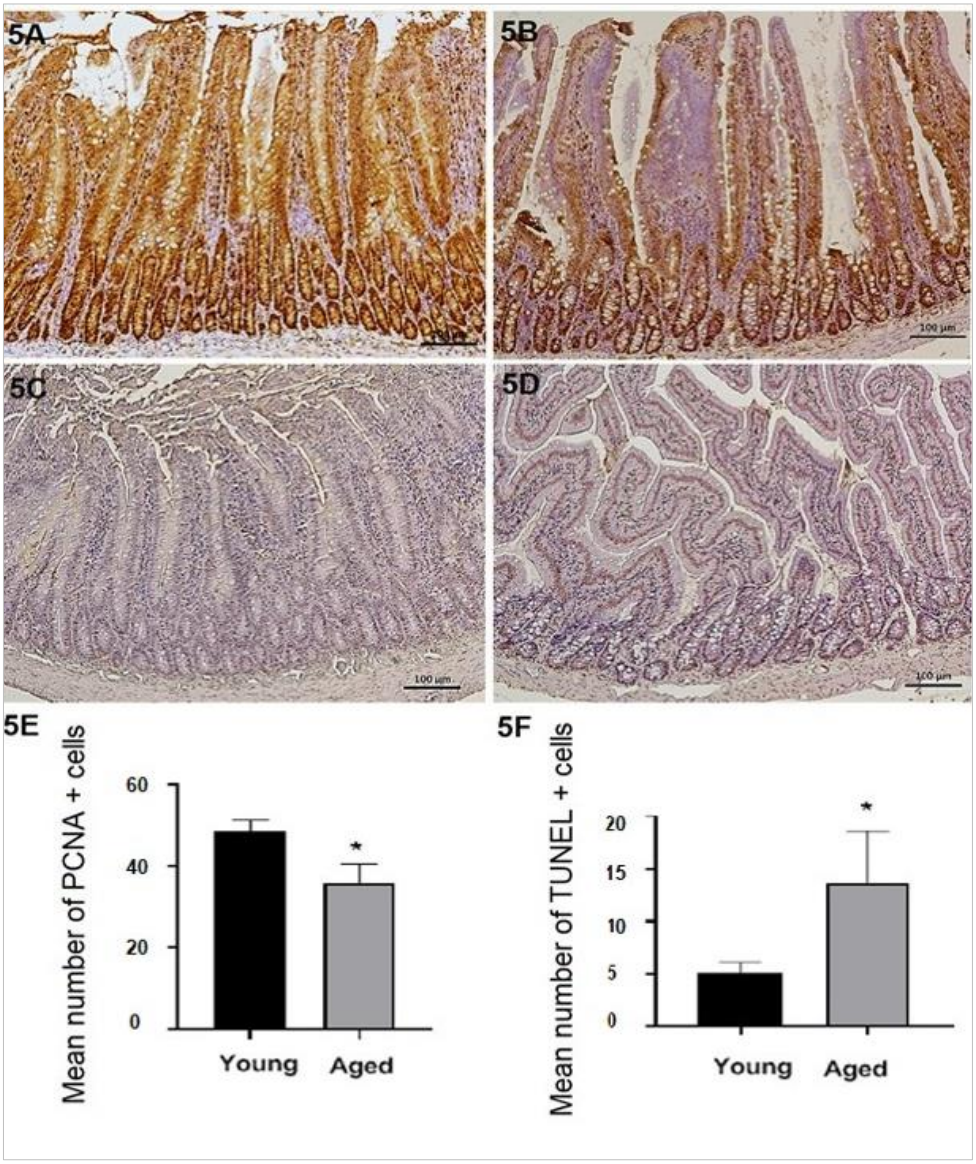

Figure 5: Proliferation and apoptosis status of jejenum epithelium of young rats (5A, 5C; respectively) and that of aged rats (5B, 5D; respectively). PCNA + cell density seems to be decreased whereas TUNEL + cell density seems to be increased in aged rats. Mean number of PCNA positive cells is significantly decreased in the aged group vs young group ($p<0.05$) whereas mean number of TUNEL positive cells is increased in the aged group vs young group ($p<0.05$) (2E, 2F).

Table I: Mean±SD stereological parameters obtained from the stomach sections of young and aged rats.

Groups	Thickness of mucosa (µm)	Thickness of mucus layer (µm)	Mean PCNA + cell number	Mean TUNEL + cell number
Young	274.965±23.599	6.939±1.345	13.350±4.101	1.675±0.512
Aged	223.435±35.294	4.894±1.328	4.325±0.411	8.375±2.575

Table II: Mean±SD stereological parameters obtained from jejenum sections of young and aged rats.

Groups	Villus length	Crypt depth	Mean goblet cell number at villi	Mean goblet cell number at crypts	Mean PCNA + cell number	Mean TUNEL + cell number
Young	127.234±13.427	277.664±23.874	13.667±1.852	11.400±2.000	48.533±2.715	5.100±1.00
Aged	234.738±38.804	71.676±17.972	24.233±5.903	13.560±3.119	35.867±4.670	13.700±4.892

Discussion

Ageing is associated with increased incidence of various diseases including digestive, cardiovascular, respiratory diseases and cancer. Mild physiological changes related with the digestive system might lead to consequences creating great disadvantages in ageing person. For instance, aspiration pneumonia due to impairment in swallowing mechanism is more common in ageing people [11]. Older individuals are particularly susceptible to malnutrition, postprandial hypotension, constipation, and fecal incontinence. Decrease in the number of ganglion cells of the myenteric plexus and degeneration of villi may lead to insufficient absorption of nutrients [5]. The relative slowing of gastric emptying resulting in the reduction of appetite and energy intake potentially contribute to wight loss called 'anorexia of ageing' [12,13]. There are numerous comorbidities which increase in prevalence with advancing age including frailty, type 2 diabetes, and neurodegenerative diseases such as Parkinson's disease [5]. In the course of ageing the main goal of the life strategy should be to slow down the development process of the system changes that are predicted to develop due to ageing and to keep the changes to a minimum level. For that purpose, to comprehend the ageing-related alterations in the wall of the organs and mechanisms

leading to these changes is crucial. In the present study we tried to investigate ageing-related histopathological gastric and intestinal alterations with light microscopic investigation.

Increasing drug use, nutritional changes, stress and changing physiology cause structural and functional changes in the stomach in ageing. Altered gastric microbiota, reduced mucosal protective mechanisms, decreased gastric blood flow, and compromised repair mechanisms are the hallmarks of age-related gastric changes [13,14]. The present light microscopic study revealed mild structural changes including increased vascularization and congestion especially at the bottom of lamina propria and increased density of connective tissue of lamina propria. On the other hand, mean thicknesses of mucosa ($p<0.05$) and surface mucus layer were decreased the epithelial barrier and mucus-bicarbonate-phospholipid barrier are crucial for gastric mucosal integrity. The first line of mucosal defense is provided by the mucus gel barrier [15]. In aged rats decrease in bicarbonate and prostaglandin secretion and thickness of mucus layer have been reported [16,17]. Age-related decreases in prostaglandin and bicarbonate secretion were also detected in older individuals [18,19]. Insufficient synthesis and secretion of prostaglandins which stimulate gastric mucus and bicarbonate secretion results in decrease in the thickness of mucus layer. It is obvious that in aged rats, mucus barrier is not as powerful as in young animals. Besides, reduction in the thickness of the mucosa represents mainly decrease in the length of the tubular glands located in lamina propria. Luminal mucus is the product of both surface epithelial cells and glandular mucous cells. Decrease in total area of gastric glands due to narrowing of mucosa is naturally associated with the decreased number of glandular cells including glandular mucous. PAS-stained sections of aged rats revealed the loss of glandular mucous neck cells. Thus, we suggest that decreased prostaglandin secretion as well as relative loss of glandular cells explain thinning of surface mucus layer indicating poor mucosal barrier at aged animals.

Gastric surface epithelium like any type of epithelium protects the mucosa from external harmful agents. Although epithelial cells are capable of mitosis under normal conditions, re-epithelialization of gastric surface epithelial cells occurs rapidly in the case of injury and subsequent damage or injury [15]. Ageing is associated with lower capacities of cellular proliferation and repair in the gastric mucosa [20,21]. The pathways involving mitogenic and cell-renewing role of two factors, epidermal growth factor and transforming growth factor-alpha has been found impaired on older rats [22]. In the present study proliferation rate of surface epithelium of aged rats was lower than that of the young rats ($p<0.05$). Instead, apoptosis rate of surface epithelium of aged rats was higher than that of the young rats ($p<0.05$). Lower repair ability of gastric epithelium of aged animals has been attributed to increased apoptosis rate [23,6,24]. Studies have reported that decreased blood flow and impaired oxygen delivery with aging lead to the activation of the Egr-1 (early growth response-1) transcription factor. Activated Egr-1 activates PTEN (chromosome 10) which induces pro apoptotic proteases [24,23]. It is clear that cell survival index (proliferation rate/apoptosis rate) of aged rats is lower than that of the young rats.

In the present study Hematoxylin and eosin-stained jejunum sections of aged rats revealed changes in the length of the villi and depth of the glands. Mean length of the intestinal villi of aged rats was higher than that of the young rats whereas mean depth of glands of aged rats was lower than that of the young rats ($p<0.05$). According to the aging study on different rat stains of Suzuki et al. [25] length of villi might be increased or conversely decreased. The length of villi has been found increased in aged mice [26] and humans [27]. The increased length of villi can be considered as a consequence of adaptation effort against nutrient deficiency. Some controversies exist in terms of ageing-related changes in depth of intestinal glands. Baum et al. [28] and Moorefield et al. [29] detected no aging-related changes in crypt depth in dogs and rats; respectively. However, Trembley et al. [30] and Nalparaddey et al. [31] reported increases whereas Suzuki et al. [25] reported decreases in accordance with our study. Intestinal crypt epithelium hosts various cells including enterocytes, goblet cells, M cells, Paneth cells and enteroendocrine cells conducting various important functions. Decrease in the depth of the crypts is expected to be associated with decrease of the number of the cells thus impairment of the functions of these cells. In this study we calculated the number of mucus secreting goblet cells in the villus and crypt epithelium. We detected ageing-related increases instead of decreases both in the epithelium of villi ($p<0.05$) and of the glands. Some controversial results exist in terms of ageing-related changes in the number of goblet cells. Mabbot et al. [32] claimed no significant change whereas Sovran et al. [26] reported decreases in aged animals. Increased goblet cell density has been reported in the intestines of 12-month-old rats [33]. Some other studies similar to the present study have reported increases in goblet cells with ageing [31,30,34]. Because of the increases in villus length, increases in the number of goblet cells of the villus epithelium is expected in aged rats. However, despite of decreases in the depth of the intestines, increases (although not significant) in the number of goblet cells in the epithelium of the glands is not expected. Similarly, Wang et al. [35] reported increases in the number of villus goblet cells and crypt goblet cells from new-born to 1st year old period.

Health of intestinal mucosa is maintained by the constant renewal of cells and also cell loss. Age-related morphological changes in the intestine have been previously determined to be the result of increased apoptosis [35] and decreased mitosis [29] as expected. In the present study, total number of PCNA + cells was decreased whereas total number of TUNEL + cells was increased in aged animals ($p<0.05$). Gradual accumulation of molecular damage during ageing may evoke a range of cellular responses such as growth arrest and eventually cell death by necrosis or apoptosis. Increased cell death may induce stem cell activation and resulting cellular proliferation. Nevertheless, some studies revealed no significant changes in terms of proliferation and apoptosis rate between young and old rodents. For instance, Turnbull et al. [36] calculated average number of S-phase and M-phase cells as well as caspase-3 + cells in the crypts of young and aged mice. They detected no significant differences between young and old group.

Conclusion

We tried to examine ageing-related histological changes in the stomach and small intestine at the light microscopic level. The advantage of working with the experimental animal model in ageing is that the effect of environmental factors (smoking, narcotic drug use or the organs harm use drug) are minimized or absent. Although conflicting results have been obtained from different experimental species, it is clear that ageing is associated with changes in thicknesses of the layers of the wall of stomach and jejunum and also in the number of cells, proliferation rate and apoptosis rate.

Declarations

All the stage of the experiment were performed in accordance with the guidelines for animal research from the National Institute of Health and was approved by the Committee on Animal Research at Bezmialem Vakıf University, İstanbul, Turkey (2013/134).

Data Availability

The data of the research is available upon request from the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

ME microscopic investigation, manuscript preparation, editing. SK microscopic investigation, manuscript preparation, stereological analysis, statistical analysis. All authors read and approved the final manuscript."

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