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RESEARCH ARTICLE

Changes in Histological Features, Apoptosis and Necroptosis, and Inflammatory Status in the Livers and Kidneys of Young and Adult Rats

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ABSTRACT

Objective: Aging entails a gradual rise in low-grade inflammation affected by cellular degeneration and death. Inflammaging refers to the chronic, low-grade inflammation that occurs alongside the aging process. This study attempts to evaluate the hepatic and renal histological changes, apoptosis and necroptosis rates, and inflammaging status of 6-week-old and 10-month-old rats.

Materials and Methods: This study uses 12 male rats separated into two groups: Young Group (6-week-old rats; $n = 6$), and Adult Group (10-month-old rats; $n = 6$). Animals were sacrificed under anesthesia. The rats' livers and kidneys were removed, and each organ tissue was divided into two parts: one for the microscopic examination (H&E and TUNEL immunohistochemistry) and the other for biochemical determination (tumor necrosis factor-alpha [TNF-α], nuclear factor-kappa beta [NF-κB], interleukin1-Beta [IL-1β], IL6, receptor-interacting serine/threonine protein kinase [RIP], and RIP3).

Results: The histological features of the livers and kidneys of the 6-week-old rats were consistent with healthy mammalian organ features, while some histological changes were detected in sections of the 10-month-old rats. The apoptosis rate indicated by TUNEL immunohistochemistry was seen to have increased in the 10-month-old rats, while the necroptosis rate indicated by RIP3 Western-blotting analysis was conversely determined to have decreased. Significant increases in TNF-α and NF-κB levels were consistent with the increased apoptosis rate in the 10-month-old rats compared to the 6-week-old rats.

Conclusion: One of the striking results of this study is that the degenerative changes related to aging began to be seen even in 10-month-old rats. The researchers used healthy rats of this age as control subjects as well as to create experimental models.

Keywords: Apoptosis, Necroptosis, Young rats, Adult rats, Liver, Kidney

INTRODUCTION

The senescence of cells is an event where cells lose their dividing ability, often triggered by factors such as DNA damage or stress. Over time, senescent cells may proliferate in tissues and contribute to aging-related illnesses and aging itself. One hallmark of cellular senescence is the development of the senescence-associated secretory phenotype (SASP), wherein senescent cells secrete a variety of inflammatory cytokines, growth factors, and proteases.[1-3] Apoptosis and necroptosis are both programmed cell death routes that are mediated by specific molecular regulators.[4] Necroptosis represents a programmed cell death pathway that exhibits features overlapping with both apoptosis and necrosis. Unlike apoptosis, necroptosis is triggered by external factors such as inflammation, infection, or cellular stress when apoptosis is inhibited.[5] Different programmed cell death pathways are interconnected on multiple levels and include shared triggers, final execution events, and cell defense mechanisms. These pathways also include the potential for one type of cell death to trigger another type of cell death in the same or different cells.[6]

Aging entails a gradual rise in low-grade inflammation affected by cellular degeneration and death. Chronic, mild-grade inflammation involving inflammaging occurs alongside the aging process. This chronic inflammation is associated with an elevated risk of several age-related diseases, including adult-type diabetes, cardiac and vascular diseases, cancer, and neurodegenerative diseases. Inflammaging can exacerbate the progression of these conditions and contribute to their development by promoting tissue damage, impairing cellular function, and disrupting normal physiological processes. This inflammation is thought to be a result of various factors, such as the accumulation of cellular damage over time, dysregulation of the immune system, and the presence of aging cells.[7-9] In humans, inflammaging is marked by the elevation of circulating pro-inflammatory cytokines, notably interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-alpha (TNF-α). These cytokines play

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crucial roles in regulating the inflammatory response and are associated with various age-related diseases.[7,9,10]

This study attempts to evaluate the hepatic and renal histological changes, apoptosis and necroptosis rates, and inflammaging status of 6-week-old and 10-month-old rats. It also aims to discover the beginning of aging-related changes in rats to make suggestions about which age periods of animals should be preferred for yielding more realistic results. The experimental studies carried out so far have investigated age-related histological changes in much older rats than this study has used.

MATERIALS AND METHODS

Animals and Experimental Groups

This study includes 12 male Sprague-Dawley rats that were formed into two groups: Group 1 (Young Group), and Group 2 (Adult Group), each consisting of 6 rats. The rats in the Young Group are six weeks old, and the rats in the Adult Group were 10 months old. The animals were sacrificed under anesthesia. The abdominal cavities of the rats were opened, and the livers and kidneys were rapidly removed. Each liver and kidney tissue were divided into two parts, one for the microscopic examination and the other for the biochemical identification. The Animal Care and Experiment Committee of Bezmialem Vakif University School of Medicine approved the animal care and research procedure (Approval No. 2024/10).

Microscopic Evaluation

Light Microscopic Examination

The kidney and liver tissue samples were fixed in 10% buffered formalin. Following fixation, the tissue samples were dehydrated, cleared with xylol, and finally embedded in paraffin. For microscopic evaluation, sections measuring 5 µm in thickness were stained using hematoxylin-eosin (H&E) and Masson's trichrome techniques. Subsequently, the samples were inspected under a light microscope (Nikon Eclipse 920248, USA).

Immunohistochemical Staining

Sections were stained to detect apoptotic cell death using a TUNEL-based apoptosis kit (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, S7101, Millipore, Darmstadt, Germany). The TUNEL technique was applied in accordance with the manufacturer's instructions. Two blinded reviewers counted the number of TUNEL-positive cells from five randomly selected fields for each sample. This counting process was conducted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) under a microscope at 20X magnification.

Western Blotting

Kidney and liver tissues were dissected, complemented with phosphate-buffered saline (PBS) that included a protease inhibitor cocktail, homogenized, and centrifuged at 9,000 x g for 15 min at 4°C. The western blot method was performed as in previous studies.[11] The study used RIP (receptor-interacting serine/threonine protein kinase) and RIP3 (receptor-interacting serine/threonine kinase 3) to indicate the presence or absence of necroptosis. IL-1β (Novus Biologicals, USA), RIP, and RIP3 (Abcam, Boston, MA, USA), TNF-α, IL-6, and nuclear factor kappa B (NF-κB; Cell Signaling, Danvers, MA, USA) were used as the primary antibodies. Signal detection was carried out utilizing a luminol substrate (Elabscience, Houston, TX, USA) and recorded using a CCD camera with the Fusion FX7 system (Vilber Lourmat, Collegien, France). Verification of protein loading was accomplished using a monoclonal mouse antibody specific to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abbkine, Atlanta, Georgia, USA).

Statistical Analysis

Statistical analysis was performed using SPSS 22.0. All data were expressed as mean ± standard error of the mean (*SEM*), and the difference between groups was analyzed using Student's t-test, with $p < 0.05$ being considered statistically significant.

RESULTS

Histology of the Liver and Kidney in the Young and Adult Rats

Various changes were detected in terms of the histological features and protein expressions related to both liver and kidney tissues between the 6-week and 10-month groups.

The H&E-stained liver sections from the 6-week-old rats appeared healthy, consistent with expectations. Classical liver lobule organization was obvious (Figure 1A). Hepatocytes possessing euchromatic nuclei and acidophilic cytoplasm containing soma basophilic granules were arranged as anastomosing cellular rows separated by sinusoidal capillaries. Periportal Kupffer cells and even bile canaliculi were observed between the adjacent hepatocytes (Figure 1B). Masson's trichrome-stained liver sections revealed collagen fiber organization. A thin layer of collagen fibers surrounded the thin-walled central vein (Figure 1C) and also observed within the portal space (Figure 1D). The H&E-stained liver sections from the 10-month-old rats were generally healthy, as expected. However, their sinusoid capillaries looked narrower than those of the 6-week-old rats (Figure 2A). Some deeply acidophilic stained hepatocyte groups were also interspersed among the healthy hepatocytes (Figure 2A). The nuclei of some of these hepatocytes seemed normal, while others were darker or paler than those of the

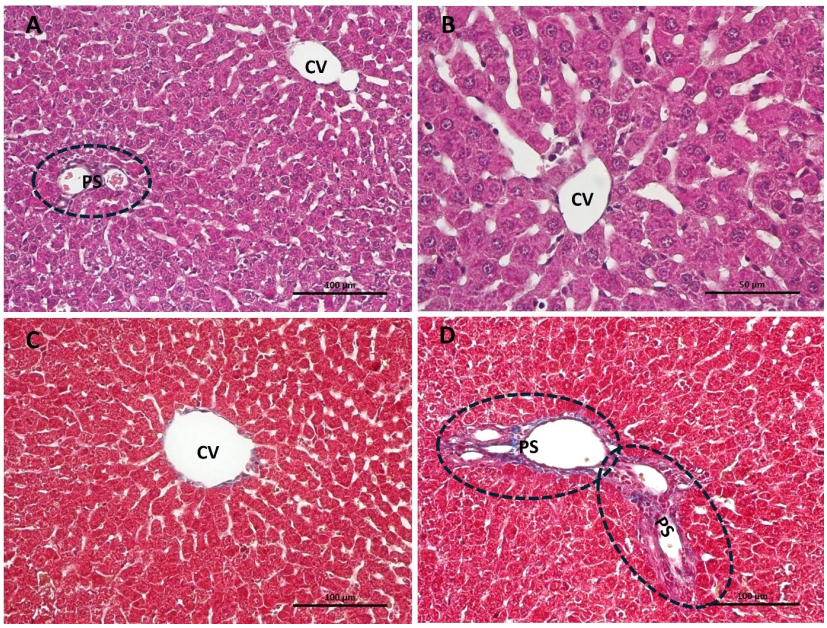

Figure 1. Histological features of the liver from 6-week-old rats. Central veins (CV) and portal spaces (PS) are regularly organized (A and B). A thin layer of collagen fibers surrounding central vein (C) and within the portal space (D) is observed A and B. Hematoxylin and Eosin; X20 (A) and X40 (B); Masson's trichrome; X20 (C) and X40 (D).

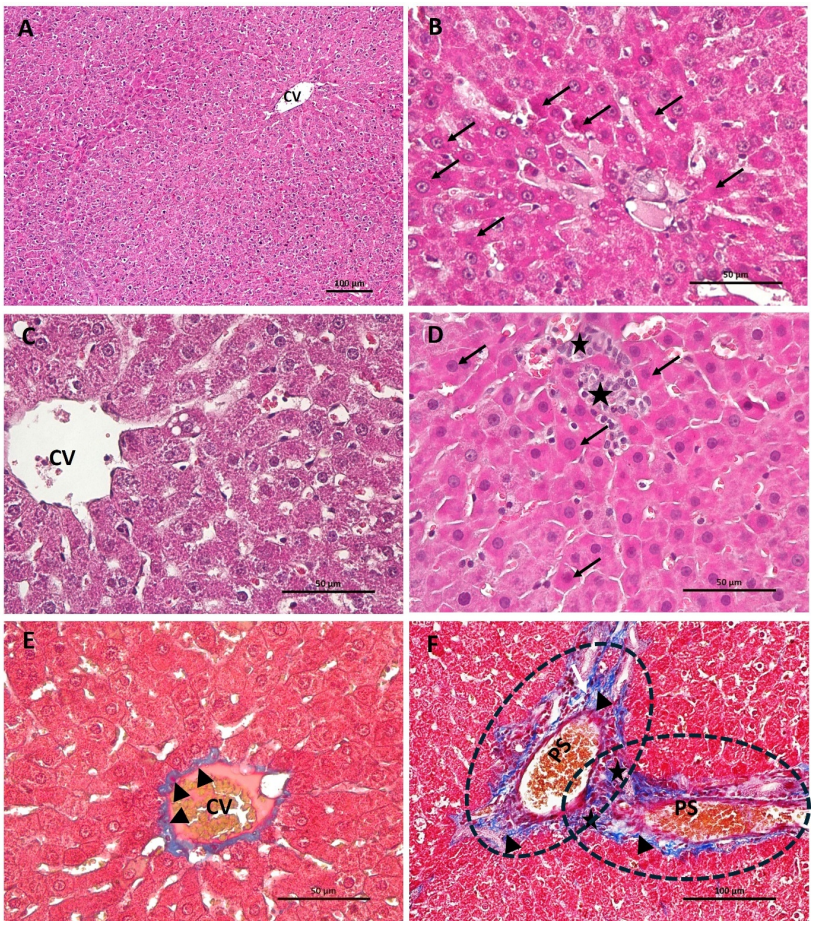

Figure 2. Histological features of the liver sections from 6-week-old rats. Deeply acidophilic stained hepatocyte groups are obvious among the regular hepatocytes (arrows) (A, B and D). Cytoplasmic vacuolization is evident both in deeply acidophilic stained hepatocytes (A, B and D) and healthy-appeared hepatocytes (C). Small inflammatory cell groups (black stars) are seen among deeply acidophilic cell groups (D) and within the portal spaces (PS) (F). The collagen fiber mass (marked by black arrowheads) encompassing the central vein (CV) (E) and within portal space (F) seems thicker than those of the previous group. Hematoxylin and Eosin, X20 (A), X40 (B) and X40 (C). Masson's trichrome, X40 (D) and X20 (E).

healthy hepatocytes. Additionally, cytoplasmic vacuolization was evident in both the apparently healthy hepatocytes (Figure 2B) and deeply acidophilic stained hepatocytes (Figure 2C). Occasionally, some small inflammatory cell groups were detected, especially among these cell groups (Figure 2D) as well as within the portal spaces. Masson's trichrome-stained liver sections revealed the collagen fiber mass around the central veins (Figure 2E) and within the portal spaces (Figure 2F) to be higher than those in the 6-week-old rats.

The H&E-stained kidney sections of the 6-week-old rats were healthy, as expected. Many glomeruli were detected surrounded by Bowman spaces in the cortex of the kidneys, as well as proximal and distal tubules, also as expected (Figure 3A). At higher magnifications, the macula densa, both layers of the Bowman capsule, striated border, and even basal striation of the proximal tubule cells became obvious (Figure 3B). Masson's trichrome-stained kidney sections revealed collagen fiber organization. A thin layer of collagen fibers was seen to surround the parietal layer of the Bowman capsule, but was only barely seen between the tubules (Figures 3C and 3D). The H&E-stained kidney sections of the 10-month-old rats were generally healthy, as expected. However, contraction of the Bowman space and tubule lumen was obvious in some places (Figure 4A). Under higher magnifications, the presence of some deeply acidophilic stained epithelial cells was detected in both the proximal (Figure 4B) and distal tubules. Regularly stained tubule epithelial cells also showed some abnormalities, including edema and heterochromatin condensation (Figure 4B). Interestingly, this group was detected to have some metaplastic changes related to the Bowman capsule. The simple squamous epithelium of the parietal layer of the Bowman capsule had transformed into a simple cuboidal epithelium, similar to that of the proximal tubule that included basal striation (Figure 4C). Some small inflammatory cell groups were detected in the interstitial area (Figure 4D). Masson's trichrome-stained kidney sections revealed the collagen fiber mass around the Bowman capsule and among the tubules (Figures 4E and 4F) to be higher than those in the 6-week-old rats.

IL-6, IL-1β, RIP3, RIP, NF-κB, and TNF-α Levels in the Young and Adult Rats' Liver and Kidney

RIP3 expression in the liver tissue was higher in the young rats compared to the adult rats ($p < 0.005$; Figure 5A). RIP expression was also higher in the young rats, though it did not reach the accepted level of significance (Figure 5B). The expressions of NF-κB and TNF-α were significantly higher in the adult animals compared to the young animals ($p < 0.05$ and $p < 0.005$, respectively; Figures 5C and 5D). In addition, no difference was found between the young and adult rats regarding IL-6 expression (Figure 5E).

IL-1β levels in the kidney tissue were notably higher in the young rats compared to the adult rats ($p < 0.05$; Figure 6A). In addition, RIP3 protein expression was slightly higher in the young rats compared to the adult rats ($p < 0.05$), whereas RIP protein expression was similar in both age groups (Figures 6B and 6C). Unfortunately, the Western-blotting protocol for NF-κB, TNF-α, and IL-6 failed despite repeated efforts.

Expression Patterns and Levels of TUNEL Positivity in the Young and Adult Rats' Liver and Kidney

The results of the mean % area measurement, which enables quantifying the percentage of tissue area that is positively stained for TUNEL, revealed the apoptosis levels to be slightly higher in the adult livers and kidneys compared to those of the younger rats (Figures 7A and 7B), though not statistically significant.

DISCUSSION

One of the fundamental mechanisms in the aging process is cellular senescence.[12] Senescent cells have a complex SASP, which includes an extension of pro-inflammatory variables with vital paracrine and autocrine impacts on cell and tissue biology. Both clinical evidence and experimental research have established connections for cellular senescence with the buildup of senescent cells and the generation and dissemination of SASP components, which contribute to age-related health issues.[13] Factors that initiate the formation of senescent cells encompass DNA damage, shortened telomeres, activated oncogenes, metabolic cues such as reactive oxygen species (ROS), physical strain, and impaired mitochondrial function.[14-17] A significant portion of senescent cells generates an SASP that is both pro-inflammatory and pro-apoptotic. The inducers, the length of time from induction, and microenvironmental factors of senescence vary according to cell type. SASP might be comprised of cytokines (e.g., IL-1a, IL-6, IL-8, TNF-α, interferon-γ) and chemokines, as well as growth factors, microRNAs, ROS, and exosomes.[18,19] Aging is characterized by an increase in mild chronic inflammation known as inflammaging, which is acknowledged as a crucial factor in the initiation of aging-related diseases.[20] In humans, inflammaging is defined by elevated levels of circulating pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β.[8] Although the current study detected no excessive pathological inflammation, hepatic expressions of NF-κB and TNF-α were significantly higher in the adult rats compared to the young ones.

In laboratory settings, Sprague-Dawley rats typically reach sexual maturity and adulthood at around 6-8 weeks of age. Adult life can be considered to begin around this age, as this is when they reach physical and sexual maturity. As for the onset of old age in Sprague Dawley rats, it generally starts around 18-24 months of age, at which point these rats may start exhibiting

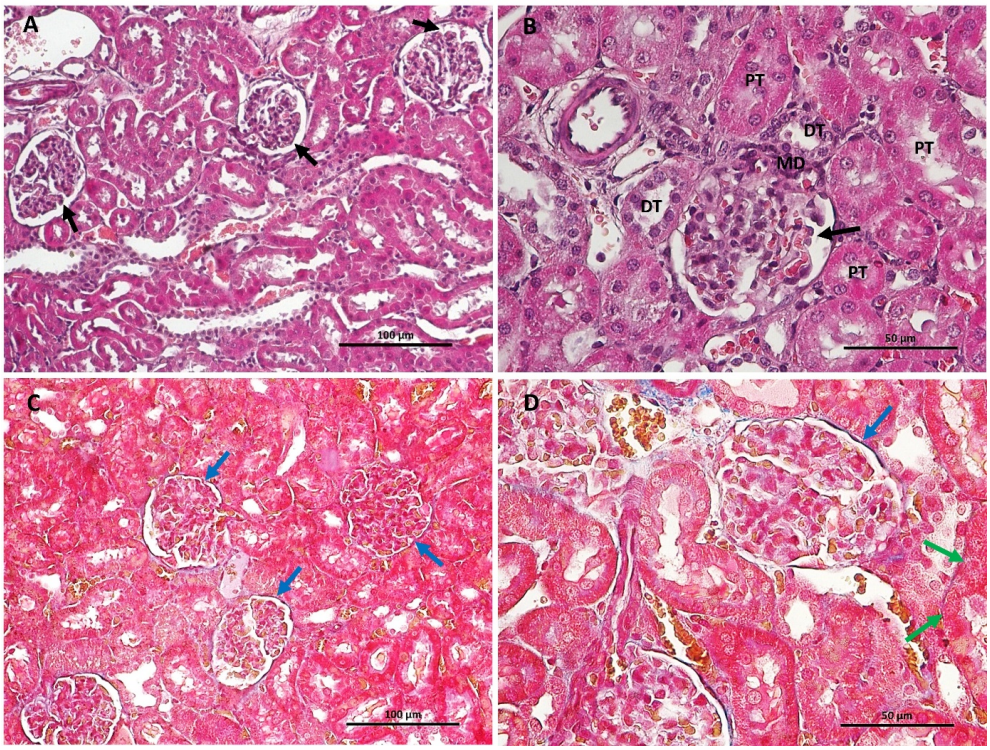

Figure 3. Histological features of the kidney sections from 6-week-old rats. Many glomeruli surrounded by Bowman spaces (black arrow), and proximal (PT) and distal tubules (DT) are detected in the cortex (A-D). Macula densa (MD), both layers of the Bowman capsule, striated border, and basal striation of the proximal tubule cells are seen (B). A thin layer of collagen fibers surrounding the parietal layer of the Bowman capsule (blue arrow) is seen (C and D). Note that collagen fibrils are barely seen between the tubules (green arrow) (D). Hematoxylin and Eosin, X20 (A) and X40 (B). Masson's trichrome, X20 (C) and X40 (D).

signs of aging. Due to both 8-month-old and 10-month-old rats typically being considered adults in the context of rat development, researchers generally use 8- or 10-week-old rats as subjects without hesitation, regardless of the age of the animals held by the animal research center. However, even the category of so-called adult rats can still have differences in cellular features, proliferation, and apoptotic, necrotic, or necroptotic cell death inflammation, organ morphology and physiology, and susceptibility to certain conditions. Consistent with this observation, while the histological features of the livers and kidneys of this study's 6-week-old rats are considered consistent with healthy mammalian organ features, some histological changes were detected in the sections of the 10-month-old rats. For instance, the presence of deeply acidophilic stained hepatocyte groups, vacuolization, and small inflammatory cell groups was evident in the liver sections. The presence of some deeply acidophilic stained epithelial cells was also detected in the kidney sections of this group in both the proximal and distal tubules, as well as cellular edema, nuclear heterochromatin condensation, and metaplastic changes related to the Bowman capsule. Also, some small inflammatory cell groups were observed in the kidney sections. In addition to the histological changes, the study also detected some differences in terms of the rates of apoptotic and necroptotic cell death and inflammatory mediators. The TUNEL immunohistochemistry indicated the apoptosis rate to have increased conversely, the RIP3 Western-blotting analysis indicated the necroptosis rate to have decreased in the liver and kidneys of the 10-month-old rats. The levels of NF-κB and TNF-α expressions in the liver samples were notably elevated in the 10-month-old rats compared to the 6-week-old rats. As age advanced, the inflammatory cytokine levels in the liver were found to increase.[21] Inflammation and cell death are interrelated processes with significant overlap. Inflammatory signals can induce various forms of cell death, and cell death, particularly necrosis and necroptosis, can trigger and sustain inflammatory responses. Additionally, inflammation often results in the production of reactive oxygen species (ROS), which can cause cellular damage and death through oxidative stress mechanisms. Cytokines such as TNF-α, IL-1, and IL-6, produced during inflammation, can directly induce cell death. TNF-α, for instance, can trigger apoptotic pathways in certain contexts, leading to cell death. NF-κB, a key transcription factor activated by inflammatory signals, can regulate the expression of both pro-apoptotic and anti-apoptotic genes.

Regulated cell death modalities include apoptosis and necroptosis, which are mediated by dedicated molecular machines, and play a significant role in health and disease.[4] Numerous levels of connections exist among various forms of regulated cell death. These encompass shared molecular pathways such as common triggers, final execution steps, and cellular defense mechanisms. Moreover, the potential exists for one type of cell demise to induce a different type of cell death in the same or other cells.[4] Necroptosis could be considered the best-characterized form of regulated apoptosis.[22] Apopto-

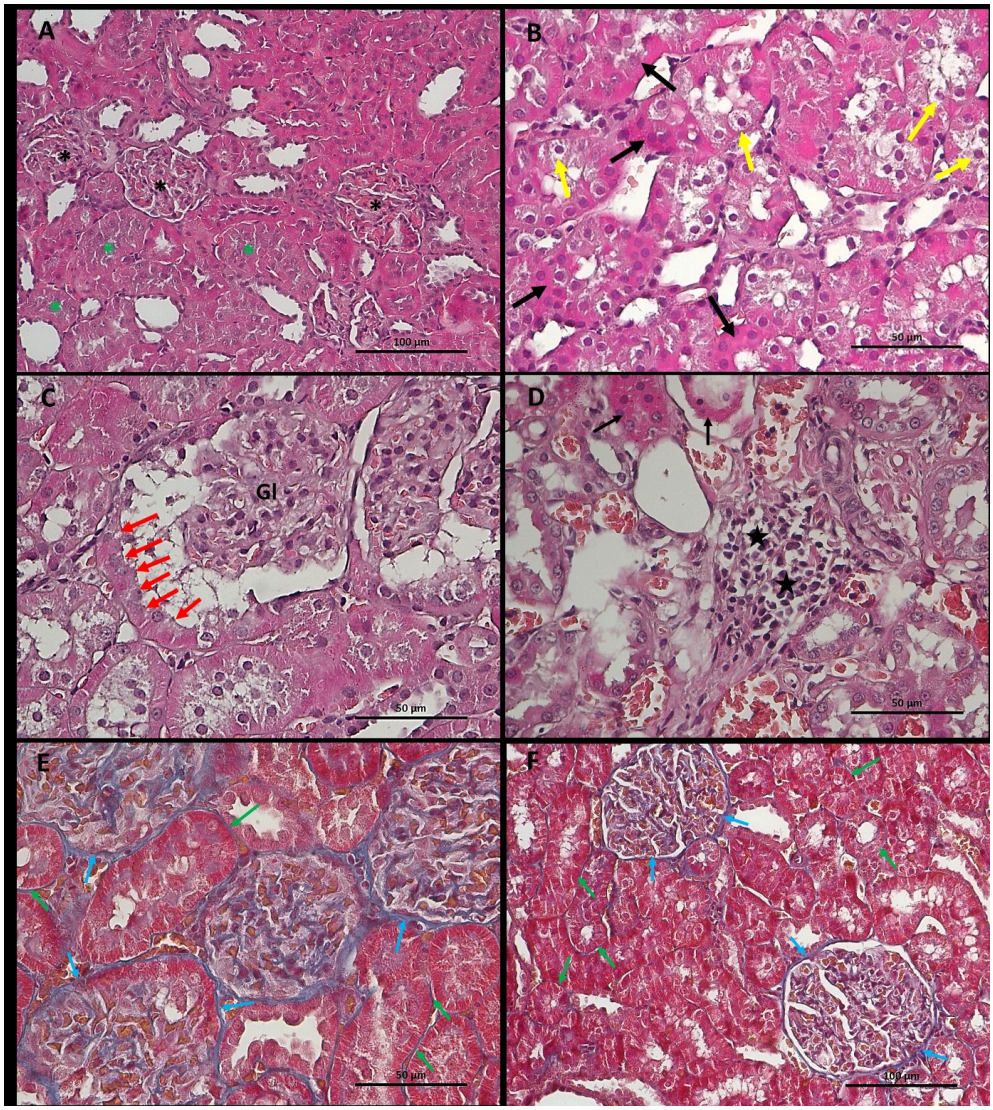

Figure 4. Histological features of the kidney sections from 10-month-old rats. Bowman spaces (marked by black asterisks), and the lumen of tubules seem contracted (marked by green asterisks). Some deeply acidophilic stained epithelial cells (black arrow) are detected within the tubular epithelium (A-D). Edema, and heterochromatin condensation (yellow arrow) are obvious within the tubular epithelial cells (B). Simple cuboidal epithelium similar to that of the proximal tubule (marked by red arrows) instead of simple squamous epithelium is detected in the parietal layer of Bowman capsule (C). Some small inflammatory cell groups (black star) are observed in the interstitial area (D). Collagen fiber mass around the Bowman capsule (blue arrow) and among tubules (green arrow) seems higher than those of the 8-month-old rats (E, F). A-D: Hematoxylin and Eosin, X20, X40, X40 and X40, respectively. E and F: Masson's trichrome, X20 and X40, respectively.

sis is triggered by signals of death, which can originate from either within the cell (intrinsic) or from external sources (extrinsic). Cellular stresses such as DNA damage, low oxygen levels, and oxidative stress stimulate the intrinsic pathway of apoptosis. These stimuli alter the cytoplasmic dynamics to promote pro-apoptotic factors, leading to the release of mitochondrial pro-apoptotic factors such as cytochrome c into the cytoplasm. Following this, the release of endonuclease G operates within the nucleus to facilitate DNA fragmentation and nuclear condensation. The initiation of apoptosis through the extrinsic pathway entails signaling via transmembrane receptors belonging to the TNF receptor family. Signaling through TNF receptor 1 leads to the activation of initiator caspase-8, ultimately resulting in apoptosis.[23] Being a pleiotropic cytokine, TNF-α induces a diverse array of cellular reactions, spanning from inflammation and proliferation to programmed cell death.[24] TNF-α has been identified as capable of directly instigating two forms of cell death: apoptosis, which is dependent on caspase activity, and necroptosis, which occurs independently of caspase involvement.[25] In various cell types, the inhibition of caspases fails to prevent cell death following TNF-α stimulation but instead triggers an alternative form of cell demise known as necroptosis, characterized by cell lysis.[24,26] Necroptosis is triggered when receptor-interacting protein kinase-1 (RIPK1) binds to RIPK3, prompting its oligomerization and autophosphorylation. Subsequently, the active RIPK1/RIPK3

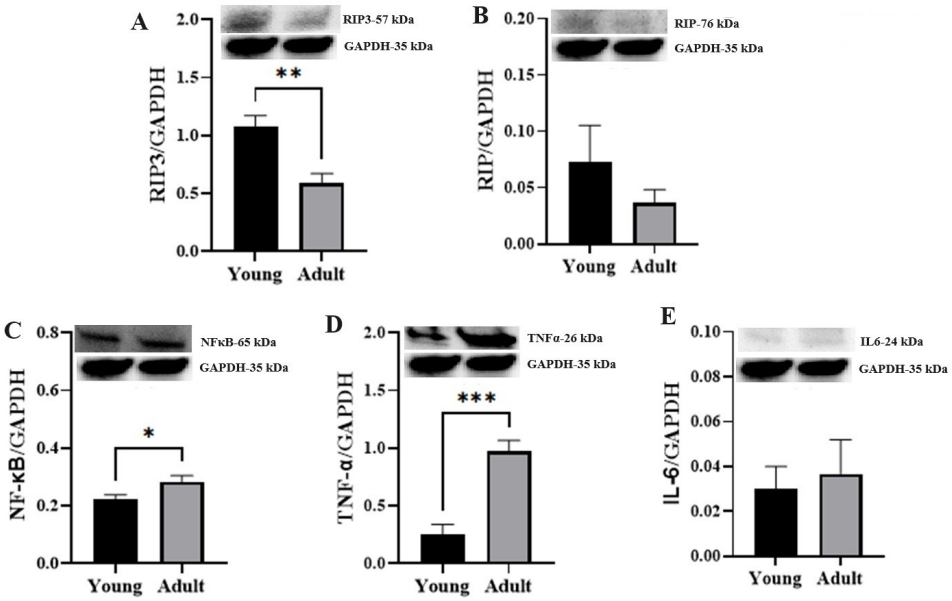

Figure 5. Semiquantitative analysis of (A) RIP3 to GAPDH, (B) RIP to GAPDH,(C) NF-κB to GAPDH, (D) TNF-α to GAPDH, and (E) IL-6 to GAPDH in the liver tissue by Western blotting for the young (n=6) and adult (n=6) animals. Representative photos belong to the samples pooled for each group. Data are presented as mean ± SEM, and * indicates for p<0.05, ** indicates for p<0.01, *** indicates for p<0.001.

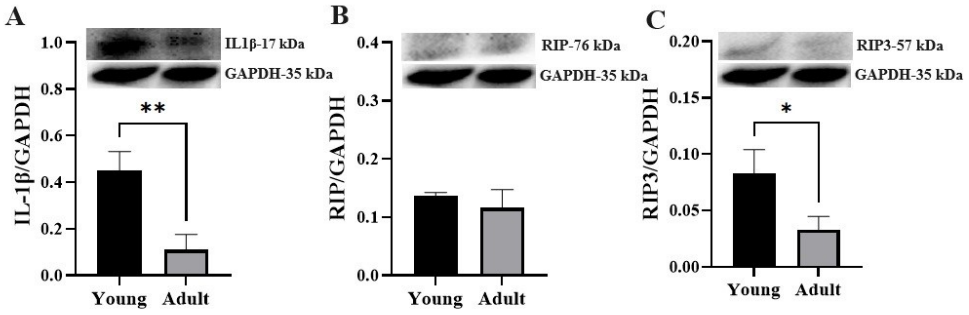

Figure 6. Semiquantitative analysis of **(A)** IL-1β to GAPDH, **(B)** RIP to GAPDH, and **(C)** RIP3 to GAPDH in the kidney tissue by Western blotting for the young (n=6) and adult (n=6) animals. Representative photos belong to the samples pooled for each group. Data are presented as mean ± SEM, and * indicates for p<0.05, ** indicates for p<0.01.

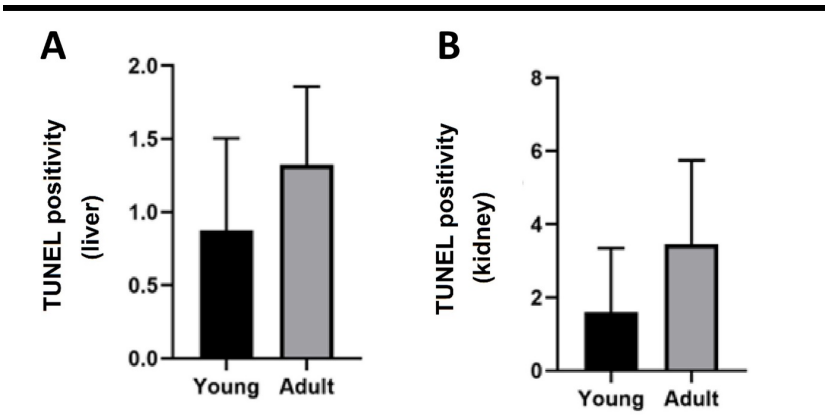

Figure 7. Expression patterns and levels of TUNEL positivity of the groups.

complex, known as the necrosome, activates the mixed lineage kinase domain-like (MLKL) pseudo-kinase protein. This activated MLKL relocates to the plasma membrane, resulting in permeabilization, rupture, and eventual death of the cell.[27-29] The binding of TNF-α to TNF receptor-1 instigates the formation of complex I, comprised of RIP1, cellular inhibitor of apoptosis proteins 1/2 [cIAP1/2], linear ubiquitin chain assembly complex [LUBAC], and inhibitor of nuclear factor-κB (IκB) kinases [IKKs]. This complex then stimulates the activation of NF-κB, leading to the induction of prosurvival genes.[30,31] Significant increases in TNF-α and NF-κB levels are consistent with the increased apoptosis rate the in liver 10-month-old rats compared to the 6-week-old rats in the current study. On the other hand, the study accumulated no signs of an aging-induced increased necroptosis rate. On the contrary, RIP3 levels in both the kidney and liver samples were significantly lower in the 10-month-old rats than in the 6-week-old rats, signifying decreased necroptosis rates. Mohammed et al. observed substantial elevations in the percentage of liver cells undergoing apoptosis and late apoptosis/necroptosis among rats over the age of 12 months.[32] Stahl et al. noted a close age-related correlation between necroptosis and liver inflammation, with both escalating notably in the latter stage of life at approximately 18 months of age, coinciding with the onset of certain age-associated pathologies in mice.[21] Marquez-Exposito et al. found no differences in gene and protein levels in the kidneys of healthy C57BL/6 mice compared to younger ones. Under the current study's experimental conditions, no age-related activation of necroptosis was observed.[22]

Apoptosis, or programmed cell death, can be both a natural and pathological process in aging rats, depending on the context and extent of the process. Apoptosis is a natural process that helps maintain tissue homeostasis by eliminating old, damaged, or unneeded cells. As rats age, there is a natural increase in the rate of apoptosis in some tissues as part of the aging process. This helps remove cells that have accumulated damage over time, which can prevent the onset of certain age-related diseases. When apoptosis occurs excessively, it can lead to the degeneration of tissues. In aging rats, this can contribute to the deterioration of vital organs and systems. In the current study slightly increased TUNEL positivity indicates aging-induced physiological apoptosis.

CONCLUSION

In conclusion, one notable finding from this study is that degenerative changes related to aging began to be seen even in 10-month-old rats. Healthy rats of this age were used as control subjects and to create experimental models. Both situations may have had the effect of unknowingly changing the results of the study. In most cases, even if researchers identify some slight histopathological or biochemical changes in the samples of control groups, these go ignored because they are considered unnatural. Because aging-related changes begin around 10 months, this study recommends researchers to use much younger rats in their studies to avoid unsubstantial results.

Ethics Committee Approval: This study was approved by the ethics committee of Animal Care and Experiment Committee of Bezmialem Vakif University School of Medicine approved the animal care and research procedure (Approval No. 2024/10).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.R.H., M.E.; Data Acquisition- E.R.H., S.K.; Data Analysis/Interpretation- E.R.H., M.E.; Drafting Manuscript- E.R.H., M.E.; Critical Revision of Manuscript- E.R.H., M.E. ; Final Approval and Accountability- E.R.H., M.E., S.K.

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