YASIN ALI HASSAN

HISTOLOGICAL, BIOCHEMICAL, AND GENETIC INVESTIGATION OF LIVER AGING IN RAT

NEU 2024

NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT HISTOLOGY AND EMBRYOLOGY

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M.Sc. THESIS

Yasin Ali HASSAN

Nicosia

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M.Sc. THESIS

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IV

Approval

We certify that we have read the thesis submitted by **Yasin Ali HASSAN** titled "**HISTOLOGICAL, BIOCHEMICAL, AND GENETIC INVESTIGATION OF LIVER AGING IN RAT**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of the institute of graduate studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Yasin Ali HASSAN

…./…/2024

Acknowledgments

I would like to extend my deepest gratitude to my supervisor PROF. DR. AYSEL KÜKNER, the head of the Department of Histology and Embryology, for her unwavering support throughout my master's degree. It is because of her valuable interest in my professional career, she has profound knowledge of every subject, and she is a strong supporter without exaggeration, that profoundly influenced me both professionally and personally. She is a perfect example of a humanitarian and moral individual whose values will always inspire me.

Thanks to veterinarian MS. MELIS TEMİZEL, whose helpful knowledge and experience regarding experimental animals made it impossible to neglect her help in our experimental processes. in addition to that, I would like to extend my most incredible gratitude to MS. GAMZE KOCAMAZ, who provided dedicated support to my thesis, especially during my experiment. Furthermore, I wish to thank the great teacher and precious guide, DR. GULTEN TUNCEL, whose wisdom and support throughout this thesis have become a constant source of appreciation. Moreover, I would like to express my gratitude to ASSOC. PROF. DR. FİKRET DİRİLENOĞLU for his sincere support through my experiment.

Finally, I must acknowledge my family, who have been my backbone, their trust, support, and patience in every challenge or joy in life have been the bedrock of my success Particularly my dear father, ALI HASSAN, for his tireless support and significant contribution of my educational journey.

To each of you, I am deeply thankful.

Abstract

HISTOLOGICAL, BIOCHEMICAL, AND GENETIC INVESTIGATION OF LIVER AGING IN RAT

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June, 2024, 86 Pages

This work was done to determine the possible histological and molecular differences to be recorded in liver tissues from Prepuberty, young, and old male Wistar albino rats. The study was undertaken to evaluate structural changes and quantify the expression of some significant biochemical markers of apoptosis, autophagy, and pro-inflammatory cytokines, including caspase-3, mTOR expression, IL-6, and NF-κB. The study used Male Wistar albino rats, provided by the Near East University Experimental Animals Research Center, separated into three age groups: Prepuberty (4 weeks), young (10 weeks), and old (18 months), with six rats in each group. their livers were quickly dissected and fixed in 10% formaldehyde solution for histological preparations. Hematoxylin-Eosin, Masson's Trichrome and PAS staining to evaluate liver architecture. Blood samples were also collected for biochemical analysis. Similarly, gene expression analysis was carried out on liver samples from all birds for caspase-3, mTOR, IL-6, and NF-κB genes by PCR method. GraphPad will be used to calculate the significance of changes observed in this study. The results show age-related histological and molecular alterations of significant effect on the liver tissues. Histological examination has demonstrated the structural changes, including variations of liver architecture and cellular composition, more evident in older rats. Scoring table results, histological findings (fibrosis, vacuolization, bile duct proliferation, mononuclear cell increase, sinusoid enlargement-congestion).in addition to those Biochemical studies showed that ALT, AST, ALP, cholesterol and triglyceride have difference significances based on the aging of rats.

Keywords: Rat, aging, liver.

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List of Abbreviations

AST- Aspartate aminotransferase,

ALT: Alanine Aminotransferase,

ALP Alkaline Phosphatase

NFKB Nuclear Factor Kappa B

il-6 Interleukin-6

MTOR Mammalian Target of Rapamycin

RNA Rib nucleic acid,

cDNA complementary deoxyribonucleic acid

qPCR quantitative polymerase chain reaction qPCR

HE Hematoxylin-Eosin

CHAPTER I

Introduction and Purpose

The liver is one of the pivotal organs in the bodies of organisms, among others, detoxification, protein synthesis, and lots of biochemicals required for digestion. As organisms age, hepatocytes can undergo several structural and functional changes that have a significant effect on their performance. Such knowledge is indispensable for developing strategies for the maintenance of liver health and for the reduction of liver diseases associated with age (Stahl et al., 2018).

Histological studies, mostly at tissue or cellular levels of microscopy, show excellent views on cellular and structural changes within the liver. Biochemical reviews may point out variations in metabolic and enzymatic activities which could accompany aging (J. Wang et al., 2023). Interest has been very high with regard to the molecular mechanisms underlying such changes with specific reference to apoptosis, autophagy, and inflammation that have all been implicated (Y. Zhao et al., 2022).

Apoptosis is a defined programmed and tightly controlled death of cells for the maintenance of cellular homeostasis. Caspase-3 expression, generally termed as the key effector in the pathway of apoptosis, showed high indication of the amount of apoptosis the liver tissues have undergone. (J. Wang et al., 2023). Another important process is autophagy, driven by the mammalian target of rapamycin. This again is another key process, removing any damaged organelles and proteins and hence antioxidant activity against aging. In contrast, inflammation is often associated with the aging and chronic disease process (Y. Zhao et al., 2022). Proinflammatory cytokines include tumor necrosis factor-alpha interleukin-6 (IL-6), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappa B), among others, that are usually upregulated in aged tissue and further contribute to hepatic dysfunction (Stahl et al., 2018).

The current work aims to determine histological dissimilarities in liver tissue at the biochemical and molecular levels in prepubertal, young, and old rats. We would try to offer insights into the whole course of how aging is impacting the liver structure and function. This work will try to assess changes in liver tissue among different age groups to identify age-related structural changes. On the same point, the

determination of the expressions of the pro-apoptotic casp-3, autophagy-related mTOR, along with inflammation-related IL-6, and NF-kappa B, is crucial in the characterization of biological activities and biochemical pathways in the development of aging-related changes in the liver.

Specific aims of the study are to compare the architecture of liver tissue in prepubertal, young, and old rats to bring out specific structural changes that occur with aging; to quantify and analyze some biochemical markers in the liver tissues of the respective age groups, highlighting metabolic and enzymic changes; to quantify the expression of caspase-3 (cas-3) in the liver tissues across age groups to understand the process of apoptosis in the aging liver; and to explore the expression of mTOR in liver tissues to investigate autophagy in the aging liver, finally assessing pro-inflammatory cytokines IL-6, and NF-κB in liver tissues to understand their contribution in inflammation.

CHAPTER II

Literature Review

2.1 Liver

The liver's primary role in digestion is the production of bile, a complex chemical required for the emulsification, hydrolysis, and absorption of lipids in the duodenum. The liver is an important organ that acts as an interface between the digestive system and the circulation. It is the organ that breaks down nutrients obtained in the small intestine before they are dispersed throughout the body. Approximately 75% of the blood that leaves the stomach, intestines, and spleen enters the liver through the portal vein. The hepatic artery allows the remaining 25% of blood to enter the liver, where it supplies oxygen to the organ (Mescher, 2019).

Hepatocytes, the main cells of the liver (Greek: hepar, liver), are among the cells in the body with the widest range of functions. Hepatocytes and other liver cells digest blood components and also have an exocrine purpose in the synthesis of bile components. They have several distinct functions in this regard. Production and endocrine release of the major plasma proteins, such as transferrin, apolipoproteins, albumins, and fibrinogen, into the bloodstream. amino acid conversion to glucose (gluconeogenesis); breakdown (detoxification) and conjugation of poisons consumed, such as several medications; Amino acid deamination causes the kidneys to eliminate urea from blood (Mescher, 2019) .

2.2 Liver Physiology

Approximately 500 tasks are performed by it. In addition to producing proteins and biochemicals needed for growth and digestion, the liver also carries out detoxification processes. The primary cell constituent of it is the hepatocyte, which is used for a variety of purposes. Gluconeogenesis, glycogenolysis, and fat metabolism are other metabolic processes that are mediated by these cells. The body releases it back into the bloodstream as needed after it has been absorbed into the bloodstream and stored as glycogen in the liver. Lipoprotein synthesis, which produces lipids that

combine with proteins to carry lipids and cholesterol, is one of the other metabolic processes (Arias, 1988).

Another of the major functions of the liver includes detoxification Most of the comparatively harmless molecules that are produced when drugs, alcohol, and other toxins are broken down by the hepatocytes may be eliminated from the body. Ammonia is converted into excretable urea as an intermediate product of the metabolism of albumin and clotting proteins (Arias, 1988).

It produces bile that is stored in the gall bladder and released into the small intestine to help in lipid absorption and digestion. Iron, copper, and the previously listed vitamins A, D, E, K, and B12 are among the vitamins and minerals that are mostly stored in the liver. Blood is filtered by it (Arias, 1988).

2.3 Liver Development and Aging

The liver is among the vital organs in human beings. It begins to form early during embryonic development, and its development entails very complex processes that are regulated by numerous genetic and environmental factors. Some of its main functions include detoxification, protein synthesis, and production of chemicals required for digestion. Its origin lies in the foregut endoderm, and it undergoes a series of important morphological and functional changes (R. Zhao & Duncan, 2005).

One of the first processes in liver development is hepatic bud formation. This is initiated through crosstalk between the cardiac mesoderm and septum transversum mesenchyme, in which fibroblast growth factor and bone morphogenetic protein pathways are critical in inducing the endoderm to proliferate and differentiate into hypoblasts that is, the progenitor cells for hepatocytes and bile duct cells (R. Zhao & Duncan, 2005).

As the liver bud grows, hypoblasts begin to express liver-specific genes including those for vital functions like metabolism and detoxification. This gene expression is regulated by a network of transcription factors including HNF4α and FoxA proteins, which ensure that hepatocytes develop properly and liver architecture is set. Meantime, coinciding with this development, the liver's vascular system begins its

development to form a complex network necessary for its all-important detoxifying function (Kinoshita & Miyajima, 2002).

At the advanced phases, the liver is still differentiating and maturing. Hepatocytes develop an ability to perform greater specialized functions of storage of glycogen, production of urea, and synthesis of plasma proteins. Its architecture develops an organization with the formation of lobules which act as functional units of the liver. In fact, such processes are wound up by signaling pathways that critically control not only the proliferation but also differentiation of liver cells, including the Wnt/β-catenin pathway (Burke et al., 2018).

The histology of liver specifies hepatic lobule, portal lobule and liver acinus with the three metabolic zones. Zone 1 encircles the portal tracts and it is here that the oxygenated blood from hepatic arteries mixes in the sinusoids with blood from the portal vein. Zone 3: it is located around the central vein where the amount of oxygenation is much less; zone 2 lies between zone 1 and zone 3 (Morsiani et al., 2019).

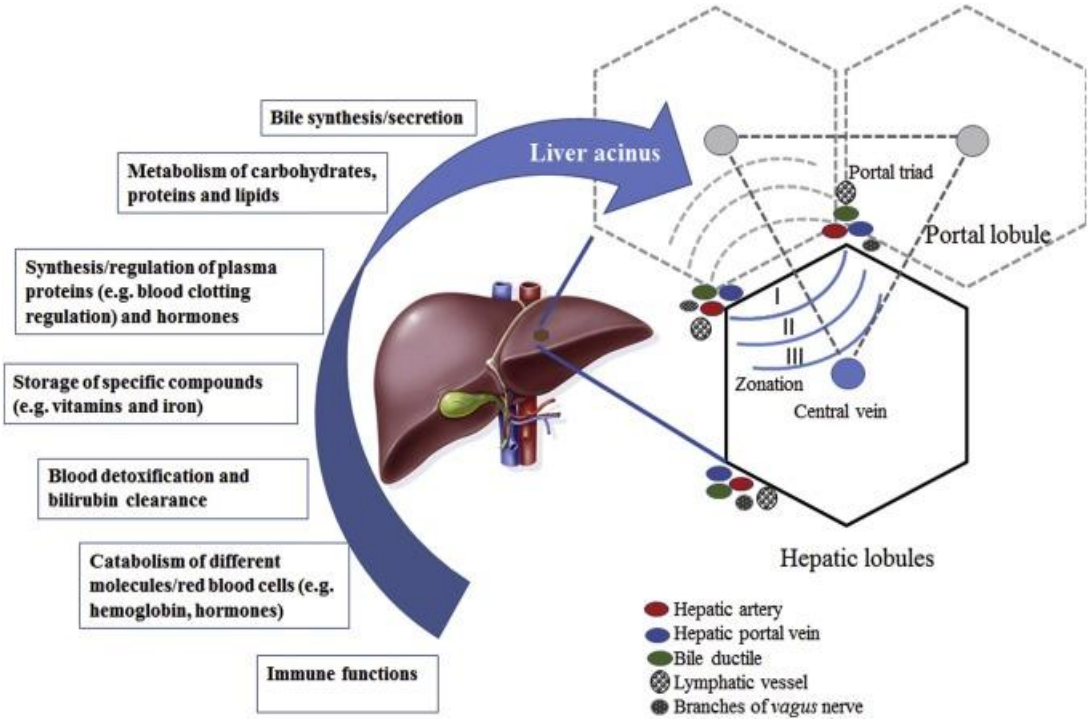
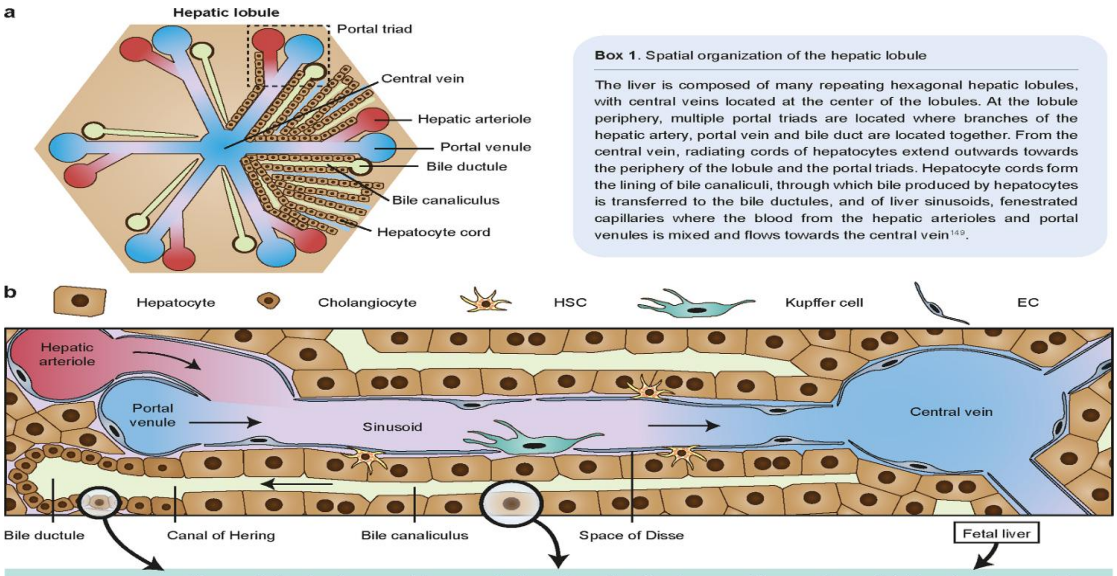


Figure 1. Liver functions and structures (Morsiani et al., 2019).

The hepatic lobule is the working part of the liver and is designed in an exquisite architecture to allow for its many metabolic, detoxifying, and immunological functions. A schematic of the portal triad within the liver lobule is shown here opposite to the central vein to display the flow of blood from the periphery of the lobule to the center. This design permits the effective transfer of chemicals between blood and liver cells. The hepatic stellate cells (HSC) and endothelial cells (EC), involved in potential functions associated with liver fibrosis and vascular integrity, respectively serve as principal scaffolds for the hepatic cords projecting from the central vein; these cords are covered by hepatocytes and interspersed with sinusoidal capillaries (Peng et al., 2021).

An in-depth look at the hepatic lobule reveals the diversity of liver cell types, from specialized cells such as HSCs or endothelial cells to significant hepatocytes and choanocytes cell types that each play some role in the (Peng et al., 2021).



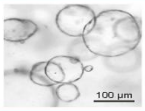
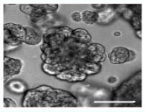
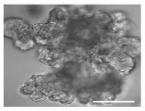
	Human cholangiocyte organoids Huch *et al.*, 2013; 2015[20,21]	Murine primary hepatocyte organoids Peng *et al.*, 2018[35]	Human fetal hepatocyte organoids Hu *et al.*, 2018[35]
Morphology			
Expansion medium	EGF, HGF, FGF10, WNT-CM, RSPO1-CM, NOG, GAST, A83-01, FSK	EGF, HGF, TNF-α, CHIR, A83-01, NOG	EGF, HGF, FGF7, FGF10, TGF-α, RSPO1-CM, CHIR, A83-01, GAST
Differentiation medium	EGF, HGF, FGF19, BMP-7, GAST, A83-01, DEX, DAPT	EGF, HGF, A83-01, DEX	Expansion medium, OSM, DEX
scRNA seq Hepatocyte genes Cholangiocyte genes		*Alb, Cyp3a11, Apoa2, Serpinc1, Mup20, Aldob, Khk, Scd1, Bhmt, Cdo1* Absent: *Epcam, Krt7, Krt19*	*ALB, SERPINA1, ASGR1, ASGR2, AFP, RBP4, APOA2, MT1G* Partially present: *EPCAM, KRT7*
Functional assays	ALB secretion, CYP3A4 activity, LDL uptake, glycogen storage, midazolam metabolism, bile acid production, ammonia elimination	ALB secretion, CYP3A11 activity, LDL uptake, glycogen storage, functional bile canaliculi	ALB secretion, CYP3A4 activity, LDL uptake, glycogen storage, bile canaliculi, AFP secretion, A1AT secretion
***In vivo* engraftment**	Very limited engraftment in CCl_4/ retrorsine-treated Balb/c nude mice	Up to 80% repopulation in *Fah*$^{-/-}$ mice	Limited repopulation in FRGN mice

Figure 2. An overview of the liver lobule cholangiocytes or hepatocyte-derived organoids properties (Peng et al., 2021)

2.4 Histology of the Aging Liver

Histopathology of the aging liver shows various dramatic changes, which finally affect its functional status and its vulnerability to diseases. Microscopic and macroscopic changes connate with the process of aging are a reflection of the sum of the environmental influences and genetic effects on the liver structure and function (Verma et al., 2012).

Macroscopically, the aging liver often decreases in size and weight. It may be due to reduced blood inflow because of increased stiffness and decreased elasticity of the hepatic artery and portal vein. Subsequently, such vascular changes may lead to less

perfusion, which importantly affects the nutrition in hepatocytes and oxygen, and this affects liver function (Mittal, 2023).

On a microscopic level, there are typical features that define an aging liver, including a variation between hepatocytes in diverse grades of morphological changes, increasing cell and nuclear size leading to a state called polyploidy, and the accumulation of lipofuscin, a cellular degradation product, in the form of pigment granules known as lipofuscin granules. Lipofuscin accumulation is considered a marker of cellular aging and oxidative stress and is commonly referred to as wear-and-tear pigment. Also, senescent hepatocytes, in general, lose some of their regenerative capacity (W. Wang et al., 2024).

There is also disruption of the extracellular matrix together with development of fibrosis in the aging liver. This may impair blood flow and change hepatocyte function through hepatic architectural disturbance. Moreover, the healthy distribution and functioning of nonparenchymal cell populations, such as liver macrophages or Kupffer cells and stellate cells, are changed during the aging process (Delire et al., 2017) (Zhong et al., 2023).

For example, senescent Kupffer cells could impair pathogen and debris clearance, or activated stellate cells could cause fibrosis. Other changes that could be seen with age in the liver might be in the bile ducts due to alteration, like the ductular reaction and ductular reaction and decreased bile flow, which might result in cholestasis and the accumulation of toxic substances (Kundu et al., 2020).

In addition, aging results in an increased pro-inflammatory state and altered immune cell function, making the immune response in the liver with age affected and potentially prone to infections and diseases (Thomas et al., 2021).

The liver histology of prepubertal rats usually shows an intact kind of cellular architecture, with freedom from distortion for the hepatocytes and having adequate supply of the cytoplasm associated with prominent nuclei. The sinusoidal spaces are also clear and open, providing adequate and efficient circulation. In addition, the components of the extracellular matrix are quite minimal, which suggest a lack of fibrotic activity and are in good agreement with high regenerative potential at this young age (Hashish, 2016).

Moving on to young adult rats their liver architecture remains robust, but subtle signs of cellular aging might become more visible. The hepatocytes can be slightly different in size, and within the nuclei, the chromatin structure can start condensing. However, these livers are quite capable of regeneration with good cell turnover and minimum deposition of fibrosis. The vascular structure is unaltered in support of metabolic demand in the liver (De Castro et al., 2013).

In contrast, livers from old rats are typically more representative of both aging changes and those of diminished regenerative capacity. As noted, fibrotic tissue is often more notable in amount; collagen deposits become more phenomenal throughout the hepatic architecture. Hepatocyte irregularity is usually more pronounced and morphologic changes such as increased cellular atrophy or ballooning may be found. Moreover, the inflammatory response is increased as senescent cells accumulate to give way to a liver-based pro-inflammatory microenvironment. The sinusoid constriction might not just further worsen blood flow but also lead to even more functional decline (Huang et al., 2005).

Aging and liver disease old livers from animals and humans are more sensitive than young ones to injury by alcohol, drugs, and toxins. This, coupled with the increased incidence with which oxidative stress, metabolic disease, obesity, and cellular senescence exert their insults, such states then further engage pathways that drive liver pathology in the form of inflammation, steatosis, and fibrogenesis. All too often these states then engage in positive feedback loops, further driving symptoms of liver disease: In the subset of these patients, liver disease will advance towards chronic hepatitis, cirrhosis, and hepatocellular carcinoma also known as the end stage of liver disease, with transplant as the sole viable therapy. Transplant is the only viable therapy for cirrhosis, whereas it forms part of the viable therapies at the hepatocellular carcinoma stage, where it also forms part of the end stage of liver disease. The growing world population has received increased liver diseases, while with elderly patients, decreased survival post-transplantation would require further therapies to be brought on board to intervene and stop the onset of the symptoms of age-related liver disease (Stahl et al., 2018).

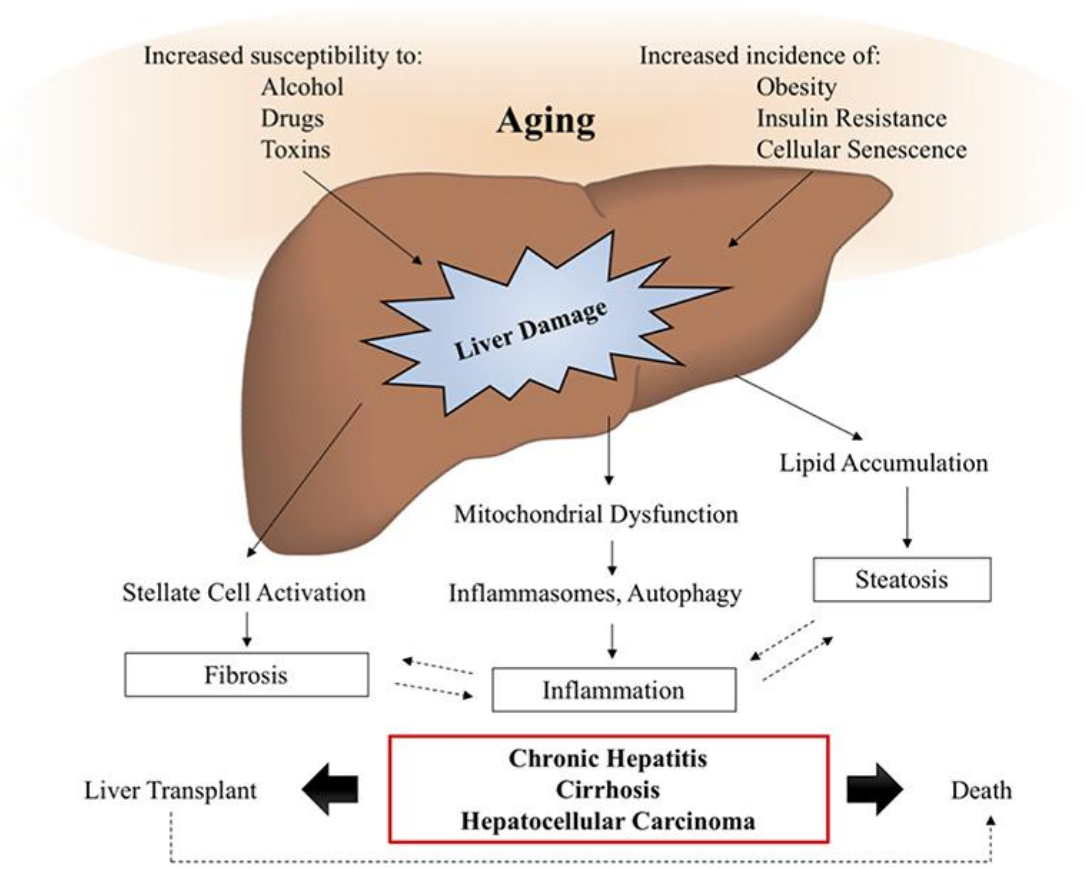


Figure 3. Both liver illness and aging (Stahl et al., 2018).

Advanced age in mice is associated with marked signs of liver degeneration, hepatic inflammation, and fibrosis. Degeneration in liver with aging also appears to be dependent on Toll-like receptor 4-mediated signaling through the increased levels of bacterial endotoxin and induction of LBP and other cascades in liver tissue. Indeed, in old age, LBP−/− mice seem to have diminished markers of senescence, thereby suggesting that bacterial endotoxin levels might have a critical effect in the aging-associated decline of liver (C. J. Jin et al., n.d.).

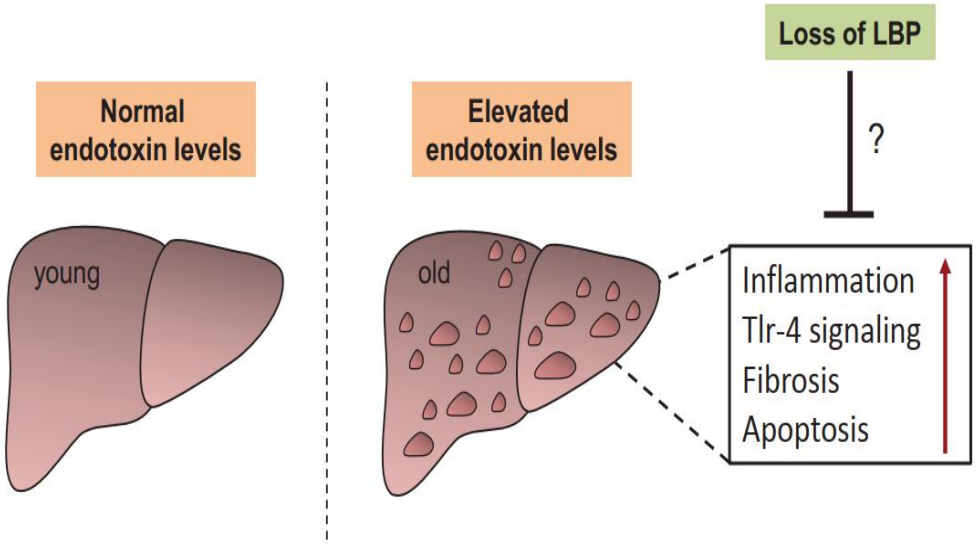


Figure 4. Degeneration of liver tissue linked to aging (C. J. Jin et al., n.d.).

2.4.1 Hepatocyte

Hepatocytes, which are responsible for most of the hepatic functions, represent about 80% of the cytoplasmic mass in the liver: metabolism, detoxification, and synthesis of proteins are some of the critical functions that place it as a very central player in maintaining homeostasis (Bhatia et al., 1996).

They metabolize fats, sugars, and proteins; detoxify a wide range of exogenous substances, including drugs and toxins; synthesize important blood plasma proteins, such as albumin and clotting factors; and produce bile, which is of utmost importance in the digestion and absorption of fats and excretion of waste products (Brosnan & Brosnan, 2009).

Remarkably, hepatocytes have an amazing ability to regenerate; they are known to divide rapidly for the purpose of repair and to restore the functionality of liver damage (Itoh & Miyajima, 2014).

More generally, with age, there are identifiable histological changes in rat models that have consequences for the functionality of the hepatocytes. Notable examples are an increase in binuclear cells and vacuolar inclusions, which may become quite pronounced under some dietary regimes or following exposure to ethanol. Further, the differences in the characteristics of the hepatocytes between the two genders blur

with increasing age. Structurally, hepatic dimension decreases, wall thickness of the hepatic artery increases, while the hepatic blood flow and bile acid secretion decreases (Kucera & Cervinkova, 2014).

Hepatocytes in the liver hypertrophy. Polyploidy (indication of double-nucleated) cells also increase; in 20-year-olds, these cells comprise 6-15%of the hepatocytes, while they comprise 25-42% in individuals aged 80 years, accordingly, signifying cellular aging and stress. In addition, aging impairs hepatic gluconeogenesis, and with an accumulation of lipids, it onsets steatosis, a process further enhanced by a decline in autophagy activity (W. Wang et al., 2024).

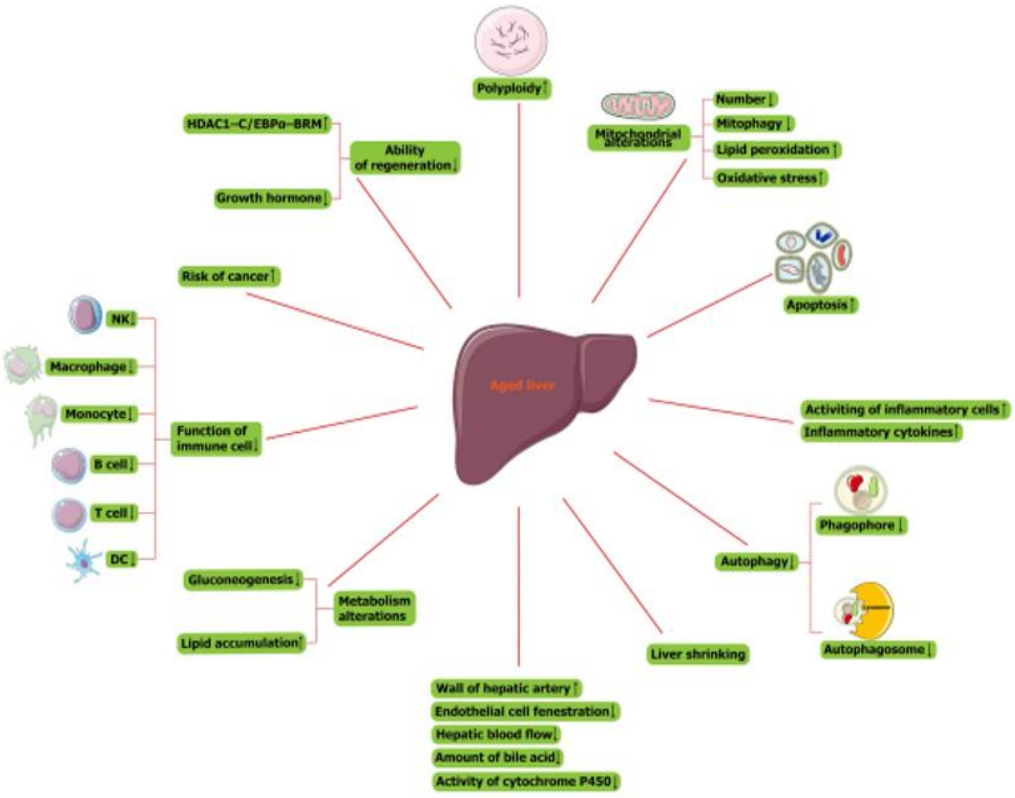


Figure 5. Aging-assoc. primary alterations in the liver brought on by natural aging (W. Wang et al., 2024).

Therefore, this improvement of autophagy is taken to be a strategy for the restoration of these age-related hepatic changes and points toward complex relationships among aging, dietary behaviors, gender, and environmental factors in the liver histology (Xu et al., 2021).

Aging is usually associated with significant cellular-level changes in the liver, reducing its metabolic and regenerative potentials.

In addition, the young liver is well equipped to allow free exchange of solutes between blood and hepatocytes, as there are plenty of fenestrations in liver sinusoidal endothelial cells (LSECs) that permit the diffusion of lipoproteins, insulin, and carbohydrates into the space of Disse. C. A potent paracrine signaling system also resides within the cell, including the primary signaling molecules, i.e., vascular endothelial growth factor (VEGF) by the hepatocytes and nitric oxide (NO) and hepatocyte growth factor (HGF) by both LSECs and HSCs. (HSCs)(Hunt et al., 2019).

Every one of these cell types in the liver changes so much with senescence, so they impair the cellular balance. For example, hepatocytes become more polyploid with higher DNA damage due to the accumulation of lipofuscin, which is often used in the literature as a marker of 'bad' aging. At the same time, it leads to an obvious decline in mitochondrial oxidative capacity, increasing oxidative stress and levels of reactive oxygen species(ROS) (Hunt et al., 2019).

A series of changes in gene expression, proteins, and metabolites could be observed during liver aging. The oxidative homeostasis has been disrupted and developed oxidative stress. Successive steps representing liver aging consist in an increased number of polyploid, senescent, and apoptotic hepatocytes. LSC from aged liver showed evidence of having more lipid droplets and released less growth factors than their young liver counterparts. Among the substantial effects of aging is the defenestration of liver sinusoid endothelial cells, which thus disrupts the exchange of substances including insulin and growth factors. In the aged liver, more extracellular matrix is also deposited in the Disse space (Y. Zhao et al., 2022).

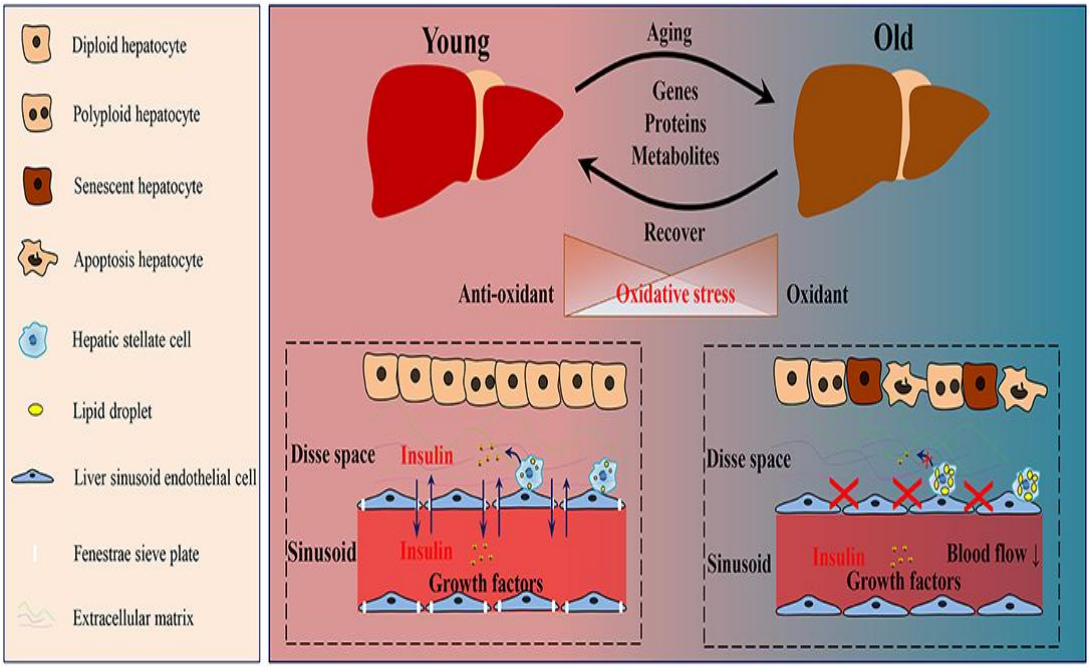


Figure 6. Diagram showing the alterations in the liver caused by aging (Y. Zhao et al., 2022).

On higher magnification, the histological images of a liver from a 6-week-old mouse show mostly normal hepatocellular architecture, with some binucleated polyploid hepatocytes. Note these particular cells with arrowheads, which are showing early development of maturity within a cell that will allow the process of regeneration and rapid capacity of cellular response (Matsumoto, 2022).

On the other hand, there is a clear increase in polyploid hepatocytes in liver from 69-week-old mouse. The nuclei are particularly enlarged, marked with arrows in the images. This increased incidence of polyploidy among hepatocytes in older mice suggests that the general strategy of cellular hypertrophy may be altered, rather than hyperplasia; perhaps this happens as a compensatory mechanism to cope with the lower capability of cellular division in aging tissues. The presence of such larger nuclei would be an indication of changes in gene expression patterns which are likely to act on the metabolic and liver function of old animals (Matsumoto, 2022).

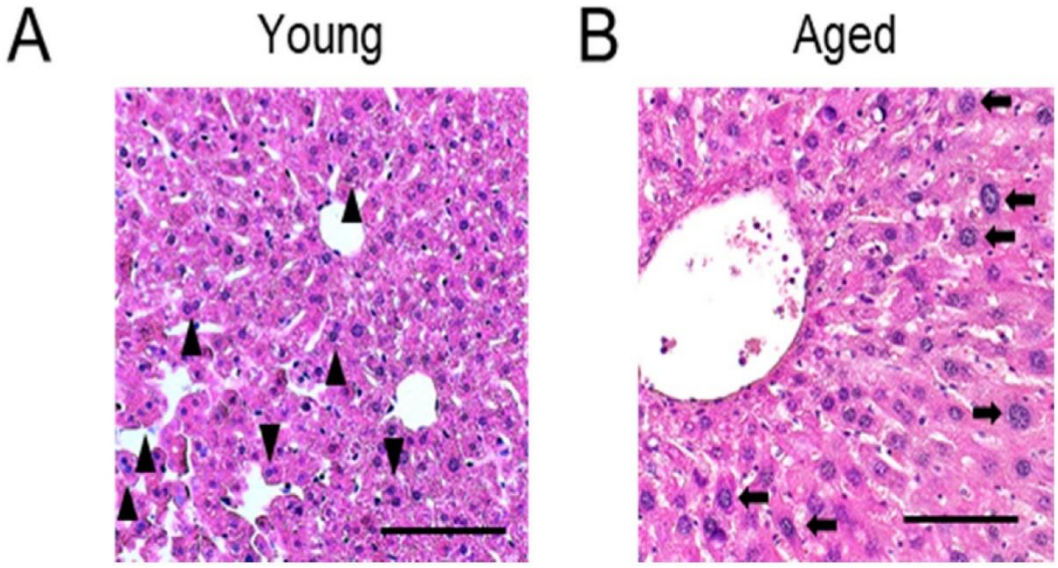


Figure 7. Mice's liver histology in young (A) and old (B) mice (Matsumoto, 2022).

Liver polyploidization is one of the more dramatic features of post-natal liver growth and reflects some of the remarkable adaptation and regenerative capacity of this organ. In this process, differentiating diploid hepatocyte will either divide to yield two diploid cells if cytokinesis is successful or it will fail cytokinesis and thus yield a tetraploid hepatocyte with two diploid nuclei. Subsequent cell cycles may lead to two mononucleated tetraploid cells or one binucleated cell with octoploid characteristics. This way, an adult liver contains a mixture of diploid, tetraploid, and octoploid hepatocytes (M. J. Wang et al., 2017).

It is a complicated process and a rigorously checked one that has a number of crucial molecular pathways. One of the most important is insulin signaling, as it impacts the metabolism of the hepatocyte and also DNA synthesis that are very important for cell division and polyploidization. Also, E2F transcription factors like E2F8 and E2F1 have vital roles in the regulation of the cell cycle and cytokinesis. It is these factors that help to establish whether a hepatocyte will be subjected to complete cytokinesis or polyploidy. Moreover, liver-specific miR-122 has been shown to be engaged in the dysregulation of such pathways controlled by p38, contributing much more to the failure of cytokinesis and polyploidization of hepatocytes (M. J. Wang et al., 2017).

In fact, the aged liver remains very plastic after transplantation, as it possesses a remarkable potential for regeneration despite advanced senescence. Much increased is the regeneration or the possibility of polyploid hepatocytes to re-enter the cell cycle after transplantation. Such reentry into the cell cycle allows these largely tetraploid or octoploid hepatocytes to expand clonally and produce a wide range of types of cells, such as mononucleated diploid and tetraploid HCCs and binucleated tetraploids through processes like tripolar or double mitosis (M. J. Wang et al., 2017).

Other evidence that supports this hypothesis is the major change in liver cellular composition, characterized by a reduction of the octoploid hepatocyte fraction and an increase in the diploid hepatocyte fraction. This essentially rejuvenates hepatic tissue or gives it a more youthful profile with respect to cellular make-up (M. J. Wang et al., 2017).

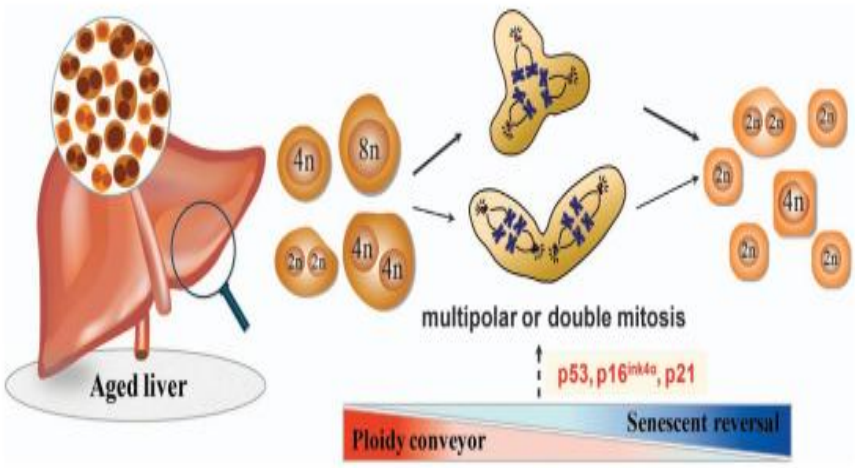


Figure 8. Ploidy Conveyor and Regenerated Senescent Polyploid Hepatocytes (M. J. Wang et al., 2017).

2.4.2 Stellate Cells

Hepatic stellate cells are the principal cells responsible for the regulation of homeostasis in liver tissue and its repair. They replenish their own tissue injury or respond to damage caused to other cells in the liver through interaction with the extracellular matrix (Kordes et al., 2021).

Hepatic stellate cells have always remained the primary focus in hepatic pathology and homeostasis in vivo and represent a cell type whose changed functionality with aging, dramatically shown by important differences in fibrogenesis between young and old rats (Kordes et al., 2021).

Stellate cells in young rats are in a quiescent state with a large pool of Vitamin A and control the ECM turnover, maintaining proper liver architecture. These are activated under conditions of acute liver damage and may lead to tissue repair and healing through the transient production of collagen and other components of the ECM (Tsuchida & Friedman, 2017) (Kordes et al., 2021).

In an older rat, though, there is the possibility of constant activation of hepatic stellate cells within the microenvironment of the liver, mediated through the level of chronic inflammation, oxidative stress, and cellular senescence associated with aging. Thereby, this leads to continued production of extracellular matrix proteins in general and collagen in particular, which are responsible for continuous deposition of fibrous tissue—the hallmark feature of fibrosis (Papatheodoridi et al., 2020) (Kordes et al., 2021)(Hunt et al., 2019)

Gene expression arrays have also revealed some insight into the functional decline that these cells exhibit over time. For instance, the expression of the senescence-associated secretory phenotype in HSCs in relation to time is linked to a concomitant reduction in the expression of the structural integrity protein glial fibrillary acidic protein (GFAP)(Kordes et al., 2021).

Part of the genes whose expression is altered comprise those involved in cell migration; thus, aged livers exhibit increased expression of both CXCR4 and MMP13 and lower expression levels of integrins anchored in their niche (Kordes et al., 2021).

Except for these genes, the studies on aged stellate cells showed that extracellular matrix-related genes and potent growth factors, such as hepatocyte growth factor (HGF), are dramatically decreased in their expression. In this regard, the drop in gene expression is very meaningful, reflecting a loss in liver regenerative potential that was initially attributed to the potent secretory and fibro-genic activities of HSCs. This reduces with aging: blood volume entering the liver and a regression of the

sinusoidal fenestrae reduces the exposure stellate cells toward mechanical stimuli (Kordes et al., 2021).

In aged rat livers, the release of HGF by stellate cells, exposed to mechanical stimuli in an integrin-α5/integrin-β1 interaction-dependent manner, is strongly impaired. This would contribute directly to a decline in the regenerative capacity of the aged livers, thus pointing to important areas in which aging disrupts liver function(Kordes et al., 2021).

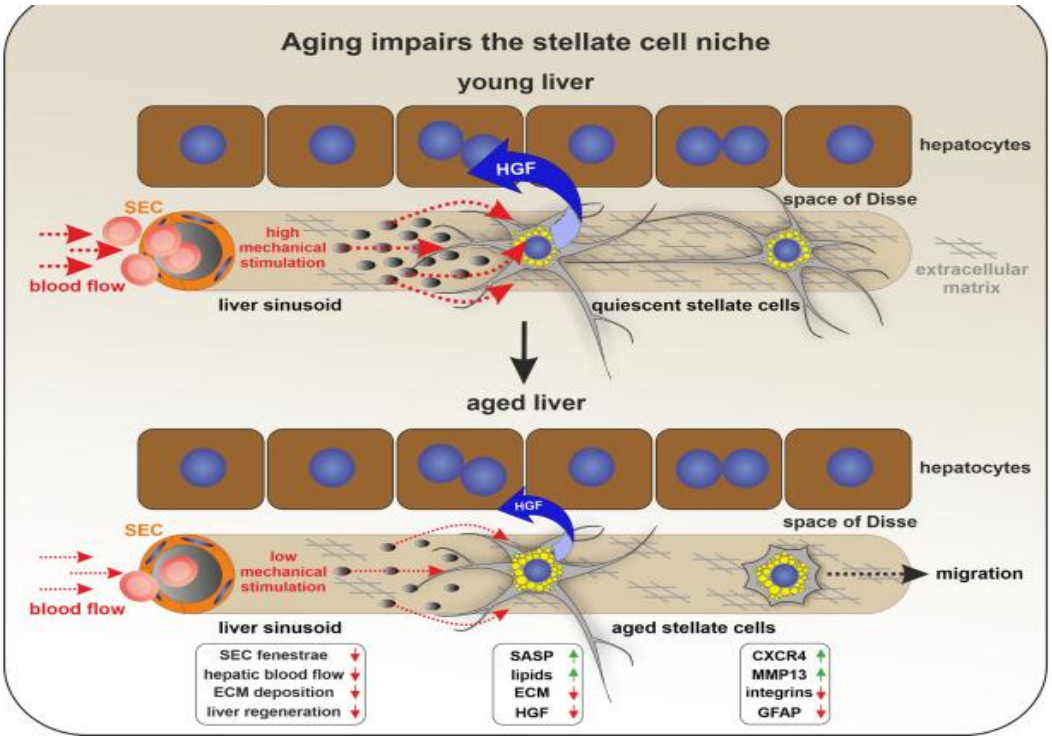


Figure 9. Stellate cell niche compromised with age (Kordes et al., 2021).

2.4.3 Autophagy and Liver Aging

Autophagy is a cellular degradation pathway that the cell employs in the removal of damaged organelles and proteins, and recycles them. Autophagy is bilevel maintenance that the liver maintains to remain in a healthy state as it grows older. The liver maintains cellular homeostasis and function by removing liver-detrimental faulty components that support body metabolism and detoxification (Profile, 2023).

Autophagy in young rats is active, and the basis for high functional activity and cellular resistance of the liver is effective. The main direction of the activity of liver cells in young rats, with regard to autophagic activity, is directed at the support of

metabolic balance and lack of overload with damaged proteins and cell organelles, respectively (H. Wang et al., 2023).

Autophagic activity declines as animals age, and it has been demonstrated in rats. The decline is associated with the onset of the mild cellular dysfunctions because the liver starts showing signs of the accumulated oxidative stress and products of lipid peroxidation. The fall in autophagy not only reduces the capacity of detoxification by the liver but also impairs its metabolic functions. This intermediate stage may not be having overt pathology but sets up the stage for more significant changes (He et al., 2024).

It is noteworthy that the features of the aging liver, such as an advanced stage of fibrosis with an accumulation of steatosis and inflammation, were associated with the failure of hepatic autophagy in aged rats. In the aging liver, in addition to the failure of the autophagic response, there is the accumulation of damaged proteins and organelles, thus reinforcing the oxidative stress and inflammation as mechanisms of histological changes in the aging liver. At this stage, there are major functional and structural impairments in the liver, usually with more severe hepatic pathology than in the earlier stages (C. Zhao et al., 2023).

It also controls the catabolism of fats, especially the turnover of mitochondria, and thus is involved in the maintenance of lipid homeostasis. The loss of capacity in lipid metabolic activity and the ability to turn over mitochondria in the liver are mainly due to the reduced autophagic capacity with age. This will further increase the lipid droplet agitation in hepatocytes, hence intensifying the production of reactive oxygen species. Elevated levels of ROS also induce more malfunction in mitochondrial functions and lipid accumulation in liver cells, thus leading to hepatic steatosis (Xu et al., 2021).

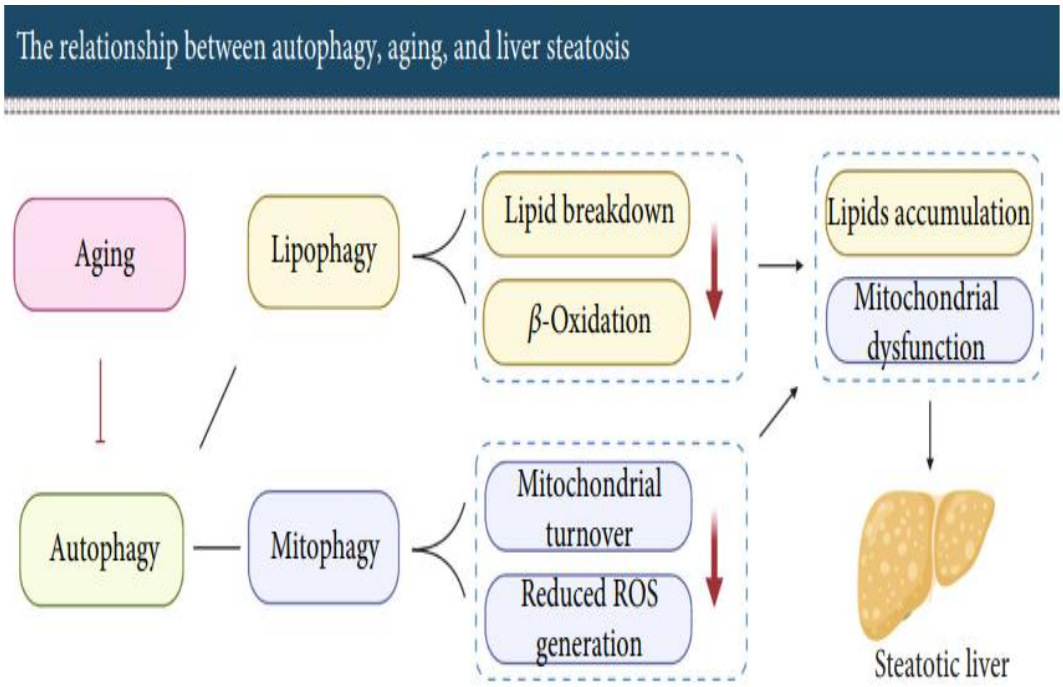


Figure 10. With age, autophagic activity decreases in the liver, which impacts the way lipids are metabolized (Xu et al., 2021).

Autophagy is set to be turning into a double-edged sword, having an imperative role to play for the development and amelioration of liver fibrosis. In addition, there are a few pro-fibro genic roles of autophagy, as it is responsible for the beginning of the fibrosis because of the supply of energy during the process of activation of the central hepatic stellate cells that are a part of the formation of fibrosis. Once activated, such cells produce an excessive matrix component giving rise to fibrosis. On the contrary, autophagy is known to be cytoprotective and increases the functioning and viability of liver cell types, including hepatocytes, endotheliocytes, and macrophages. Augmented functioning and viability of these cells help balance the process of fibrosis and maintain homeostasis in the liver (Xu et al., 2021).

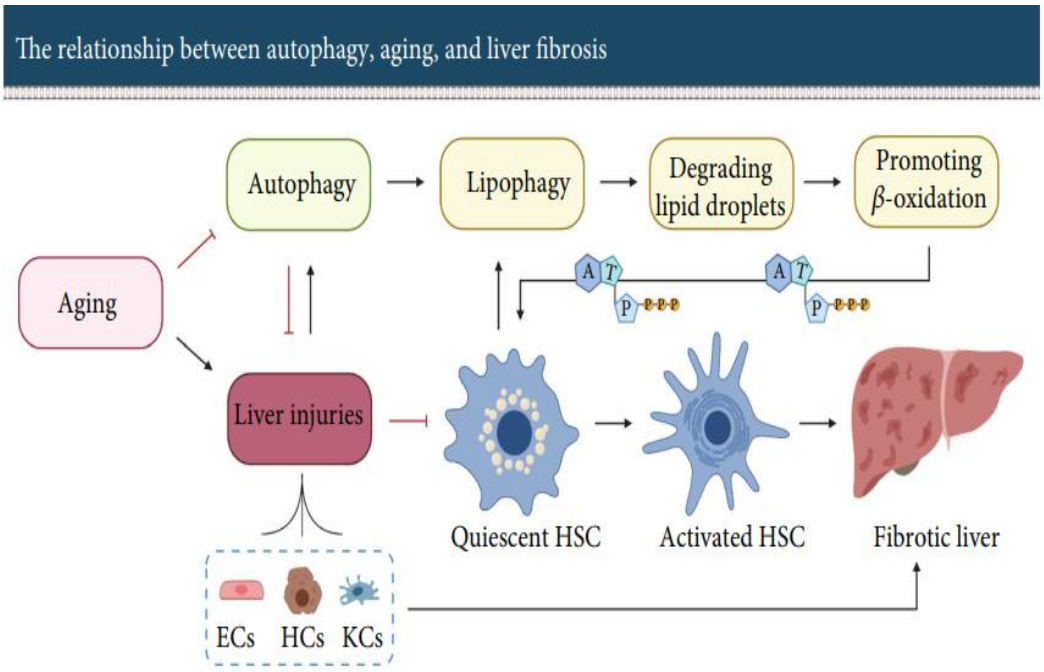


Figure 11. Autophagy has a dual role in liver fibrosis (Xu et al., 2021).

2.4.4 Genetic Factors in Liver Aging

The common histopathological and typical functional liver changes with aging are increased fibrosis, fatty deposition, and decreased regeneration. These depend substantially on genetic factors, and variation in aging processes can make this difference in histology or function between different organisms from the same species—for example, rats (Kunizheva et al., 2022).

One of those key genetic pathways involved in the biology of liver aging is the regulation of oxidative stress and mitochondrial function. For example, genes from the Sirtuin family, especially SIRT1, have been shown to play important roles in modulating these processes. For instance, in rats, a decrease in liver SIRT1 with age correlates with an increase in oxidative damage and mitochondrial dysfunction. In fact, overexpression of SIRT1 in transgenic rat models not only increases the number of mitochondria but also reduces oxidative damage, consequently preventing age-related changes (Pande & Raisuddin, 2023).

The mTOR signaling pathway is another important genetic factor in liver aging, integral in cellular growth and metabolism. Therefore, pharmacological inhibition of mTOR, or its specific genetically modified form, exerts strong effects in life

extension and significantly delays pathological age manifestations in rat liver. This inhibition leads to a suppression of hepatocyte senescence, a reduction in fibrosis, and an improvement in the functionality of these cells. It is therefore suggestive that mTOR inhibition will lead to an improvement in lifespan through such a pathway that plays a main role in cellular processes of aging. There are quite a number of evidences in literature relating the mTOR regulation of protein synthesis and autophagic processes to areas that are critically associated with the liver maintenance that goes on during aging. This further goes to point that this regulation of cellular longevity takes place through such a pathway. And so mTOR has continued to be the epicenter around which much of the research on aging circles, providing insight regarding the ways cellular metabolism drives age-related changes in liver function (Walters & Cox, 2018)(Johnson et al., 2013)(Hassani et al., 2022).

Another related process that is involved in the maintenance of liver homeostasis and function at old age is autophagy. Autophagy is an intracellular degradation process, and most of the autophagy-related genes have differential expression profiles in the aging rat liver, such as ATG7 and BECN1 (Xu et al., 2020).

Functional genes ensure the clearance of damaged proteins and organelles—a critically important task when damage is accruing over time and from oxidative damage. This crosstalk between aging and autophagy is reflected in the modulation of functional genes for autophagy (Xu et al., 2020)(Tao et al., 2023).

Additionally, genetic polymorphisms also contribute to the process of decision making over the aging course of the liver. Genetic polymorphisms may, in fact, lead to differences not only in the extent but also in the rate of progression of the liver histological changes per se, indicating that genetic background affects liver aging. APOE regarding lipid metabolism and FOXO3, the transcription factor for resistance against oxidative stress, were associated with contrasting aging phenotypes across rat populations at these loci. These polymorphic variations interact in a complex way to underlie the genetics and/or environmental factors constituting the shaping of liver aging. The findings also indicate that the genetic diversity within rat populations is, hence, a valuable resource to study biology of the aging process, and its relevancy has been supported by the fact that polymorphic changes in aging processes first

observed in rats have, in several aspects, been similar to the ones in humans (Kunizheva et al., 2022)(Hong et al., 2021).

2.4.4.1 mTOR Signaling Pathway

The mTOR pathway provides a hub for signaling in the regulation of mammalian cell metabolism, growth, and survival. Additionally, the mTOR pathway couples the morphological and physiological adaptations that occur in the rat liver during the process of aging. Again, high mTOR activity is related not only to cell proliferation and protein synthesis but also to the cellular clearance of debris and senescent cells. Controlled mTOR supports what is termed the autophagic removal of those damaged cells to sustain sufficient liver function. In this context, the deregulation of mTOR strongly correlates with liver diseases, such as fibrosis or steatosis of the aging liver (Guo et al., 2019)(Fok et al., 2014).

Aging is associated with drastic changes in the histology of the liver, characterized by the rise of fibrosis and steatosis and the reduction in cellular capacity for regeneration. All these effects seem to be explained, at least in part, by the activation of the mTOR pathway with signals from nutrients, growth factors, and the cell about the status of cellular energy to regulate growth and maintenance programs that are indispensable for the liver to fulfill in the functions that it performs. At the heart of it, the processes these pathways regulate are of cellular turnover and metabolic adaptation; both are sensitive to environmental cues through mTOR as a regulatory process of direct impact to overall liver organization and functions that tend to deteriorate due to age. Aberrant regulation or hyperactivation of this pathway directly leads to a host of common age-associated liver pathologies, including abnormal fibrosis and fat accumulation (Sapp et al., 2014).

Because it is related to accelerated aging of the liver, there is early occurrence of age-related changes in the livers of rat models. Evidence adduced from the studies has made it widely acceptable that hyperactivity of the mTOR pathway through increased protein synthesis and cellular growth and proliferation has a beneficial effect during development and repair, but, when dysregulated as in aging, it may consequently contribute to the pathological changes. For example, in this study, the exaggerated mTOR signals will be on hepatic tissue and thus speed up conditions

like hepatic steatosis and hepatic fibrosis, conditions commonly associated with aged livers (Stallone et al., 2019).

Besides, growth factors and nutrition oppose the process of autophagy, stimulate an increase in protein synthesis through activation of mTOR, and further burden the cells with oxidative stress caused by malfunctions of organelles, or even by protein aggregation. All of this will be a trend of buildup in damages, reduction in function, and possibly induction of the fatigue status in such a way that less tissue will heal and more dysfunction will be maximized. Such aging-related disorders are ruefully encouraged by the manner in which mTOR is activated (Stallone et al., 2019).

This, in turn, is regulated at the level of many cellular processes by the signaling pathway of the mTORC1 kinase. mTORC1 serves actively in the promotion of protein translation and the enhancement of the turnover of stem cells, and an increase in cellular senescence. This implies that activated mTORC1 limits autophagy, an essential process that cleanses and rejuvenates the cell. It promotes the accumulation of cellular debris and dysfunctional organelles, which, in turn, facilitates cellular aging. Some of the significant regulators of the mTORC1 are FKBP38 (FK-506-binding protein), mLST8 (mammalian lethal with sec-13 protein 8), and PRAS40 (proline-rich Akt substrate of 40-kDa), which control the magnitude of activity of the pathway and the pathway's health effects of aging(Evangelisti et al., 2016).

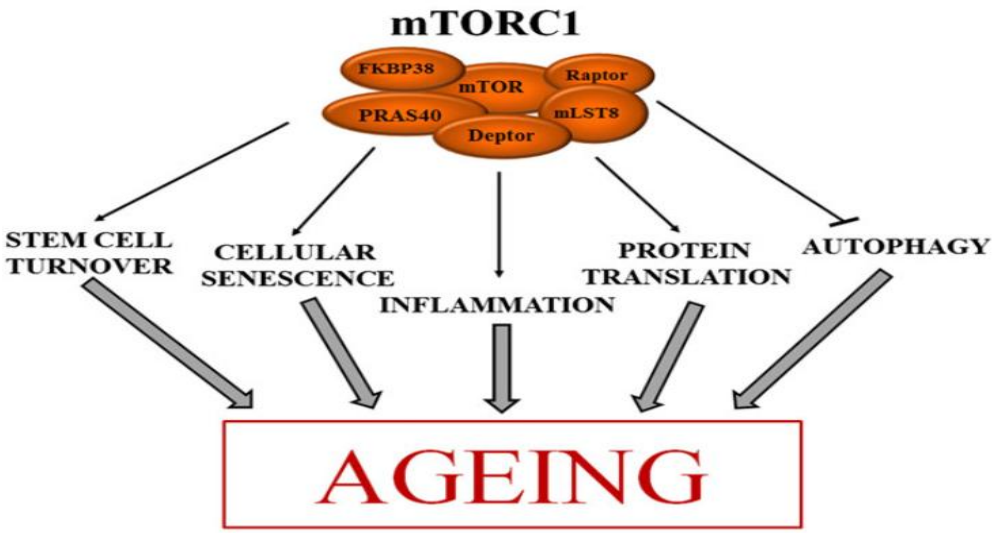


Figure 12. Influence of the mTORC1-signaling pathway on cellular aging processes (Evangelisti et al., 2016).

2.4.4.2 Caspase-3

Caspase-3 mediates apoptosis, which is important in regulating tissue integrity and cellular turnover. The liver tissue thins and the overall potential of the organ to replenish and repair itself lessens as more active Caspase-3 is observed in the liver cells, as it increases the capacity for the occurrence of apoptosis. This other accumulation of enzymes would accelerate the breakdown of cellular constituents, further impinging upon the structural and functional decline witnessed in the aging liver. High Caspase-3 activity is mainly related to high fibrotic tissues, which can disrupt the architecture of the liver parenchyma and decrease the capacity for effective functioning (Hu et al., 2019)(Shang et al., 2018).

Caspase-3 belongs to the family of cysteine-aspartic acid-specific proteases that mediate apoptosis, a form of programmed cell death considered necessary to rid the body of deleterious, senescent, or damaged cells for instance, Caspase-3-controlled apoptosis in the liver is a basis for cellular homeostasis and prevents the accumulation of potentially harmful cells that promote liver disease. Unfortunately, during the aging process, apoptotic pathways, including those mediated by Caspase-3, may become dysregulated and lead either to the excessive death of cells or to insufficient clearance of damaged cells (Ouyang et al., 2021)(Shang et al., 2018).

Caspase-3 activity is entirely regulated in young animals so the enzyme does not disturb liver function and regeneration but maintains a delicate balance between cell proliferation and cell death. However, these regulations are pretty much lost in older rats. Research has shown an upregulation of the expression and activity of Caspase-3 during the aging process, which at the same time diminishes the regenerative ability of the liver due to apoptosis. This increase in apoptosis is a response to increased oxidative stress in aging liver tissue and cellular senescence of liver cells (Liu et al., 2020)(Hu et al., 2019).

These formed reactive oxygen species are responsible for initiating damage to lipids, proteins, DNA, and other biostructures. Inflammatory and fibrotic responses develop if the injured cells are not cleared from that site. The caspase-3 has been involved in two functions during this process. One is to help the clearance of cells undergoing irreversible damage due to oxidation. On the other hand, excessive or chronic

activation of Caspase-3 can further lead to the loss of hepatocytes, which eventually thins the liver tissue and decreases the number of active hepatocytes (Aladaileh et al., 2021)(Eid & El-Shitany, 2021).

The enzymatic activity of caspase-3 in the liver catalyzes various intracellular proteins that are crucial and may affect other cellular processes. Such proteolytic activity modifies cellular signaling pathways that control liver metabolism and energy homeostasis. For example, caspase-3 action cutting into constituents of the mitochondrial electron transfer chain would disturb the normal functioning of the mitochondria, which would further upregulate oxidative stress and cell death (Guha et al., 2006).

Furthermore, the increased fibrotic activity in the aging liver is in part due to actions of Caspase-3, resulting in hepatocyte death and proliferation of the stromal cells of the liver to replace lost tissue, which further results in fibrosis (Sakasai-Sakai et al., 2017).

When the histology of the aging rat livers is performed, generally, the Caspase-3 staining is elevated, representing high apoptosis. These results are accompanied by a host of aging-related hepatic abnormalities such as hepatocellular ballooning, lobular inflammation, and steatosis—all hallmarks of the increasingly common condition of nonalcoholic fatty liver disease in older adults (C. P. Li et al., 2014).

By early adulthood, the levels of Caspase-3 expression increase. The liver consequently attains adaptation to these higher metabolic demands and increased exposure to environmental toxins, given that enhanced apoptotic activity will clear damaged or dysfunctional cells more efficiently. Through the control of regeneration concerning the removal of these harmful cells, this control helps to maintain the most optimal liver function (Z. Zhang et al., 2013).

An increase in the level of caspase-3 exposure is highly significant in aged rats and is associated with high levels of apoptosis. This age-induced increase in apoptosis is related to depressed liver regenerative potential and the accumulation of specific age-induced histological changes, including fibrosis and steatosis(Fontana et al., 2013).

2.4.4.3 TNF: Tumor Necrosis Factor

The pro-inflammatory cytokine TNF-α plays a significant role in age-related vascular endothelial dysfunction. TNF-α production depends on the stimulation of AGE-RAGE within the vasculature and the induction of the transduction of NF-κB signals. Upon increasing expression of TNF-alpha, it creates ROS, reducing the bioavailability of endogenous nitric oxide (Pomponi et al., 2010).

AGEs also accumulate in tissues through non-enzymatic glycation and oxidation of proteins and lipids. The multiligand receptor of the immunoglobulin superfamily in the human body is activated due to the accumulated AGEs, followed by a series of intracellular signaling, including induction of NF-κB, representing the transcription factor for regulating the expression of various genes related to inflammation and immune responses. Activation of inflammation-related NF-κB signaling enhances pro-inflammatory cytokines formation, TNF- α, in whose line-up is also detectable(Pomponi et al., 2010).

The increased level of TNF-α in the vasculature, aside from contributing to the production of ROS, also leads to a different type of endothelial dysfunction – impaired endothelium ability to control vascular tone and maintain homeostasis.(Pomponi et al., 2010).

The ROS are reactive molecules that affect a cell's proteins and lipids, including DNA. The generation of ROS in the vascular cell's microenvironment elicits oxidative stress that diminishes the synthesis activity of the enzyme responsible for NO synthesis in the endothelial cells. Nitric oxide in the endothelial cells is an essential ingredient required for vascular function. The enzyme that endows the capacity to generate NO is denoted as eNOS. NO generated in physiological quantities serves as a potent vasodilator and inhibits the aggregation of platelets and the adhesion of leukocytes, thereby providing for the vasculature's tone (Pomponi et al., 2010).

The increased production of ROS in the presence of high TNF-αlevels also upregulates NO breakdown, which is less biologically available. This reduction in

biological available NO stimulates endothelial dysfunction, leading to vasodilation reduction, proportionately increased vascular rigidity, and the predisposition to thrombosis and inflammation (Pomponi et al., 2010).

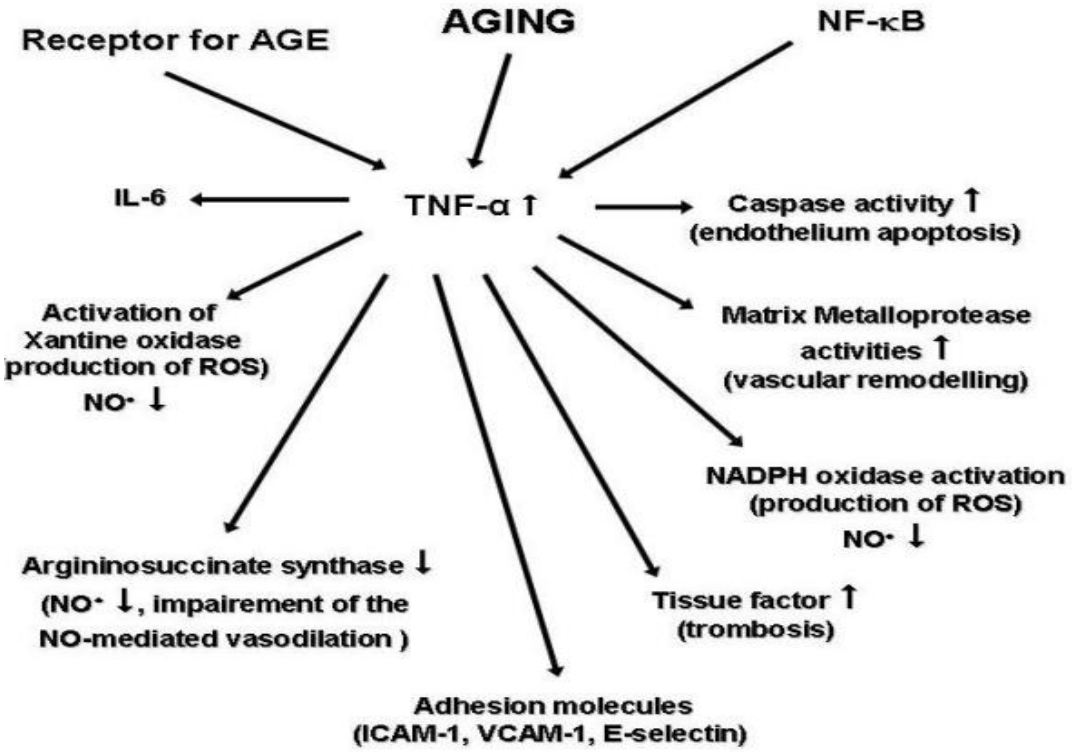


Figure 13. The function of tumor necrosis factor (TNF)-α in vascular endothelial dysfunction associated with aging (Pomponi et al., 2010).

The activation of nuclear factor kappa B (NF-κB) is directly correlated with endothelial dysfunction (Pomponi et al., 2010).

During the normal aging process, proteins undergo progressive and irreversible modifications through nonenzymatic glycation and oxidation reactions, leading to the formation of advanced glycation end products (AGEs). These AGEs accumulate in tissues over time, including within the vascular system, and their presence is associated with various age-related pathologies (Pomponi et al., 2010).

The AGEs primarily act after binding to their specific receptor, RAGE, to produce an active intracellular signaling cascade. As a result, NF-κB is activated, and there is concomitant transcription of several genes that encode the pro-inflammatory cytokines, adhesion molecules, and other mediators of inflammation. Chronic

activation of the NF-κB signaling pathway leads to a sustained, maintained inflammatory state of the vascular endothelium (Pomponi et al., 2010).

2.4.4.4 IL-6: Interleukin-6

Interleukin-6 (IL-6) releases, especially in rats, and in a highly complex way, participate in the biology of aging liver histology. It is effectuated in some cellular and molecular processes that impact liver health during aging. IL-6 is pivotal in modulating the response of the liver to fibrosis, inflammation, regeneration, and metabolic activity in the matrix. IL-6 exerts broad actions towards the liver, from essential cellular health to the development of complicated diseases such as liver fibrosis and cirrhosis (Biazi et al., 2023).

One of the significant functions that IL-6 undertakes in the liver is to be involved in its inflammatory reaction. IL-6 levels rise if inflammation develops, and this is a commonality with liver diseases Because IL-6 is involved in chronic inflammation, which is often associated with aged livers, controlling this becomes increasingly necessary as the rats age increases. This is characterized by an inflammatory disease with low-grade continuous inflammation, leading to progressive liver damage. In this mechanism, IL-6 can act as a mediator for the inflammatory response and may stimulate hepatic stellate cells as a factor determining the pathogenesis of liver fibrosis (Seki & Schwabe, 2015).

Progressive liver regeneration is another fundamental process that is impaired with aging. It supports the observation that IL-6 is required for this function in the livers of young rats after damaging stimuli. Still, this regenerative response might be impaired in old rats due to dysregulation of IL-6, leading to poor recovery from hepatic damage. This change is especially detrimental since it diminishes the capacity of the liver to regenerate by the process of healing after illnesses or physical damage, leading to a slow decline in liver function (Naseem et al., 2018).

In addition, IL-6 is also involved in hepatic metabolism regulation. It is instrumental in lipid and glucose metabolism, which is essential for the general health of the liver Dysregulated IL-6 signaling in the aging setting can predispose to metabolic diseases,

such as non-alcoholic fatty liver disease and steatosis, both common in older rats. The importance of cytokines in maintaining health during aging is shown by their participation of its in different metabolic pathways (Knudsen et al., 2016).

In addition, IL-6 is also directed towards the regulation of oxidative stress within the liver. Elevated circulating levels of IL-6 can further increase the generation of reactive oxygen species and, hence, oxidative damage; finally, this results in the oxidative degradation of the structural proteins, lipids as well and DNA. This oxidative destruction forms one of the significant causes of aging and, from there, can amplify other issues, such as increased apoptosis in liver tissues and cellular dysfunction (X. Jin et al., 2007).

Interactions of IL-6 with immune cells, including other cytokines, are, in large part, what dictates the immunological state of the hepatic milieu. Once more, these relationships may further dramatically enhance liver diseases like cancer and cirrhosis in elderly mice by invoking a more complex inflammatory response in the liver (Fontes-Cal et al., 2021).

More so, IL-6 affects liver apoptotic pathways. For instance, activation of some receptors, as well as downstream transcription factors such as the STAT3, with the raised levels of the cytokine in old rats, might lead to apoptosis (Kojima et al., 2013).

IL-6 plays a critical role in controlling liver responses toward acute, inflammatory stimuli of damage or infection in young rats. The liver needs an efficient inflammatory response for clearing infections and initiating repair processes, and this cytokine helps prepare the liver for that response. It encourages the migration of immune cells to the site of damage and instigates the production of acute-phase proteins by hepatocytes (Espat et al., 1996).

On the one hand, apparent is the ability of IL-6 expression and activity to create a paradox in aged rats: a chronic low-grade inflammation in older rats is often observable, and the levels of persistently high IL-6 are consistently associated with it. This situation does not bring similar positive effects in younger animals. On the contrary, it is involved in the manifestation of several chronic diseases of the liver,

such as hepatocellular carcinoma, steatosis, and fibrosis. IL-6 is also involved in stimulating hepatic stellate cells in aged rat livers, leading to the result of fibrosis (Gedik et al., 2005).

However, there are differences between young and aged rats in the metabolic effects of IL-6. In young rats, it takes part in regulating glucose and lipid metabolism during postprandially or reaction to acute stress, thus enhancing metabolic efficiency. In old rats, chronic signaling of IL-6 upsets metabolic balance and leads to the emergence of metabolic syndromes such as NAFLD and insulin resistance (Wallenius et al., 2002).

2.4.5 Metabolic Factors in Liver Aging

The liver undergoes drastic metabolic changes during aging, significantly affecting its regenerative capacity and functional potential. Among the derived factors, the most important involves a decrease in hepatic regenerative ability through decreasing hepatocyte proliferation and autophagy. Indeed, aged liver cells are more reactive to oxidative stress and mitochondrial dysfunction, which would additionally impede regenerative processes. This process, too, is another critical factor in the recycling of cellular components that decline with age and is one of the reasons for the accumulation of damages. However, intensifying autophagy through the mTOR-independent pathway might be beneficial to improve liver regeneration in old individuals (Xu et al., 2020).

The enzymatic systems of lipid metabolism in the aging liver also suffer from a variety of alterations, predisposing the liver to the development of hepatic steatosis and non-alcoholic fatty liver disease Insulin resistance, in combination with changes in lipid signaling pathways in aging, has the effect of worsening hepatic fat accumulation. This is further aggravated by decreased activity of enzymes within lipid metabolism, causing further disruption of lipid homeostasis. In turn, these metabolic changes not only have relevance in influencing liver function but have a systemic influence on health in a way that influences overall metabolic health in old individuals. This may be pretty relevant in twin cities, with the growing elderly population in most high-income countries (Stahl et al., 2018) (Frontiers) (SpringerOpen)(Pu & Zhou, 2022)(P. Li et al., 2021).

Another very crucial feature of aging livers is the alteration in glucose metabolism. With advancing age, glucose homeostasis is significantly lowered in the liver because of reduced cellular insulin sensitivity and the sensitivity to glucose uptake by the liver. In this way, hyperglycemia is developed and thereby induces the risk for type 2 diabetes. Age is also linked with a change in hepatic expression for essential glucose-homeostasis-regulating genes and proteins, such as those responsible for gluconeogenesis and glycogenolysis. These changes underscore the intricate cross-talk between age and metabolic processes in the liver (Pibiri, 2018).

The complex interaction between environmental and behavioral risk factors may critically modify metabolic processes, manifesting as accelerated cellular and molecular aging. In the ecological arm, one does not only consider drugs but also obesity, pollution, a low intake of plant-based ingredients, processed meats, alcohol intake, smoking, physical inactivity, and sleep apnea, all together influencing metabolic health (K. Zhang et al., 2023).

Exogenous toxins, such as drugs and pollutants, cause oxidative stress, inflammation, and derangement of normal metabolic functions. At the same time, obesity acts as an essential factor that potentiates insulin resistance and dyslipidemia, which are precursors to diabetes and cardiovascular conditions. Consumption of processed meats and other trans fats further compounds the already worsened lipid profiles and insulin sensitivity, multiplying metabolic strain (K. Zhang et al., 2023).

Behavioral factors, especially those of diet and lifestyle, play a very critical role in modulating metabolic health. The presence of low plant-based ingredients in the diet excludes important antioxidants and fibers important for the maintenance of metabolic homeostasis. Alcohol intake and smoking are potent mediators of inflammation from induction and oxidative stress, respectively, and are critical factors in the development of hepatic steatosis and cardiovascular diseases. All these problems are further compounded with physical inactivity, causing impairment in glucose uptake and lipid metabolism, thus leading to diabetes and fatty liver diseases (K. Zhang et al., 2023).

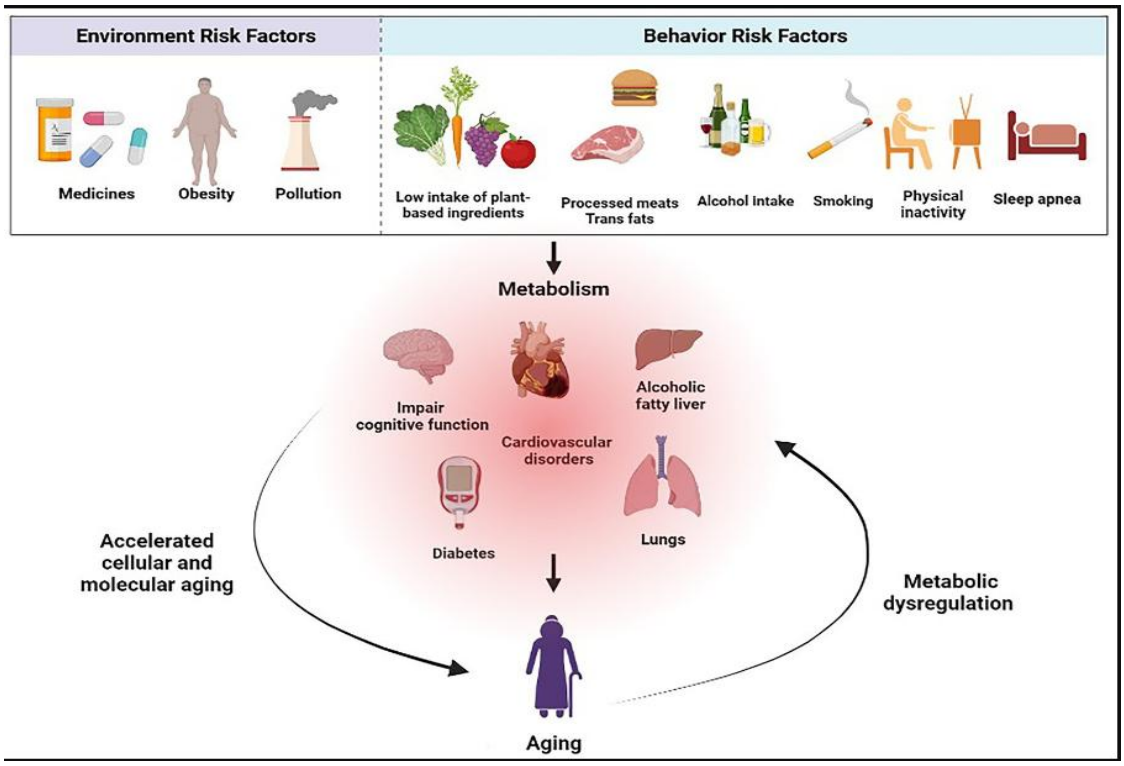


Figure 14. Linkage of the metabolic disorders and aging (K. Zhang et al., 2023).

2.4.5.1 Cholesterol in Aging Liver

The aging process is thus characterized within the liver by significant changes in cholesterol metabolism, which are reflected in the disposition of metabolic pathways as a unit. Several studies have reported that under the state of an elevated rate of cholesterol synthesis and reduced elimination, aging is associated with cholesterol homeostasis. The reduced expression of LDL receptors significantly decreases hepatic elimination of circulating LDL cholesterol and, hence, high blood cholesterol levels in the aging liver. Bile acid synthesis and secretion are among the most critical processes towards cholesterol excretion, and modifications in them further impair cholesterol homeostasis. These changes are often exacerbated by diets high in cholesterol and saturated fats, common in older people. Cholesterol aggravates these reactions (Jia, 2023)(Saher, 2023).

Another critical pathway in the metabolism of cholesterol involves the conversion of cholesterol to bile acids by the liver, a function that is impaired with aging. When little bile acid is formed, it is less effective in the emulsification and absorption of fats, which leads to hepatic cholesterol overload and hence impairs the pathogenesis

of diseases like steatohepatitis and non-alcoholic fatty liver disease (NAFLD). Such hepatic fat accumulation cholesterol metabolism association is also significant, showing the more general metabolic disturbance and dysregulation of the aging liver (Eilam et al., 2022)(Kakiyama et al., 2023).

Systemic factors impact the aging liver's metabolism of cholesterol in addition to hepatic alterations. Lipid metabolism is impacted by hormonal changes, especially the reduction in sex hormones like estrogen, which makes controlling cholesterol even more difficult. Estrogen's reduction with aging exacerbates hypercholesterolemia because it is known to upregulate LDL receptors and enhance cholesterol clearance. Besides the age related oxidative damage and inflammation, these hormonal effects add to the difficulty of controlling cholesterol levels in the elderly (Jia, 2023)

Cholesterol synthesis, absorption, and excretion are well regulated in growing livers and maintain the cholesterol balance. High levels of expression of LDL receptors ensure the effective removal of circulating LDL cholesterol in the young liver. Further, the ability to convert cholesterol more effectively into bile acids can ensure regular cholesterol excretion without accumulating in the liver and thus avoid potential health problems (Simon et al., 2023).

In contrast, this is dramatically different from cholesterol metabolism in the aged liver. With age, expression of LDL receptors is low; their down-regulation lowers the amount of LDL removed from the circulation, increasing the risk of hypercholesterolemia. The accretion of cholesterol is further aggravated with weakened synthesis and secretion of bile acids in aging livers. This is further worsened by the fact that these changes are usually paralleled by elevated production of cholesterol. As such, the aged liver is highly susceptible to diseases like steatohepatitis and NAFLD that underline metabolic imbalance associated with aging (Kakiyama et al., 2023)(Simon et al., 2023)(Saher, 2023).

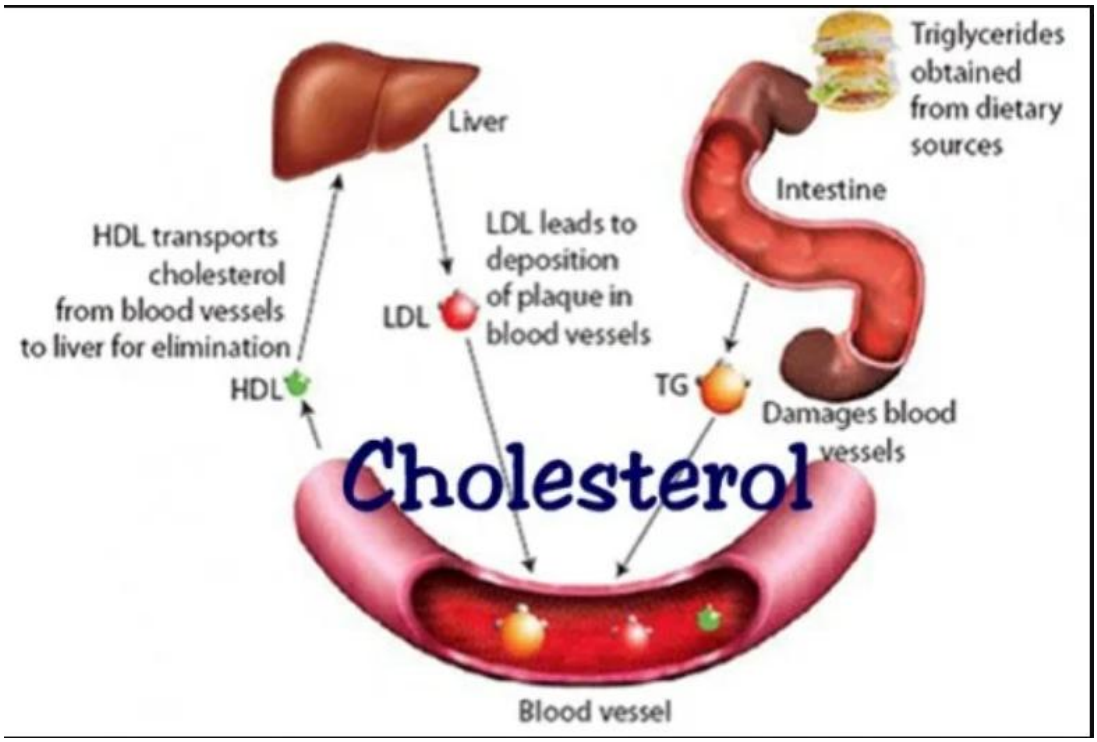


Figure 15. Disturbances of triglyceride and cholesterol metabolism and effects on blood vessels (hubpages.com/ 24 Jan. 2015).

2.4.5.2 Triglycerides in Aging Liver

Triglyceride metabolism alters significantly with ageing liver, impacting both liver function and overall metabolic health. Hepatic triglyceride buildup is linked to aging and has a role in the development of diseases like non-alcoholic fatty liver disease (NAFLD). Changes in the ratio of lipid production to clearance are the main cause of this buildup. There is a downregulation of fatty acid oxidation and an increase of lipogenic pathways in aging livers. These modifications cause triglyceride production to increase and breakdown to decrease, which encourages hepatocytes to store fat (Nunes et al., 2022)(Faquih et al., 2023).

Moreover, triglyceride buildup in the liver is made worse by insulin resistance, which frequently occurs with aging. Insulin resistance increases the amount of free fatty acids that enter the liver by impairing the liver's capacity to regulate lipolysis in adipose tissue. Hepatic steatosis is exacerbated when these free fatty acids are esterified to form triglycerides. Additionally, normal lipid homeostasis is disrupted by insulin resistance because it alters the expression of genes involved in lipid

metabolism, particularly those that control triglyceride synthesis and export (H. Li et al., 2023)(Uehara et al., 2023).

Another factor that affects the metabolism of triglycerides in the aging liver is the onset of mitochondrial dysfunction. Reduced mitochondrial activity from aging brings about oxidative stress and lowers the oxidation of fatty acids. The accumulation of triglycerides in liver cells leads to reduced fatty acid oxidation as well, which eventually leads to the build-up of triglycerides. In addition, oxidative stress is injurious to inflammation and liver injury; therefore, it promotes the accumulation of triglycerides, thereby enhancing the development of liver diseases such as NAFLD and steatohepatitis (D. Wang et al., 2023).

Young livers can balance their function for the amount of triglyceride through the oxidation of balanced fatty acid and lipogenesis. Upregulation of genes to break down triglycerides is very high in young livers, which enables the effective removal of triglycerides. Younger livers have better insulin sensitivity so that the liver can maintain the right amount of lipid homeostasis by restricting the excess input of free fatty acids. Younger livers also show vigorous mitochondrial functioning, where the chances of proper fatty acid oxidation are high, along with less oxidative stress (Lee et al., 2023).

In reality, more triglycerides are synthesized in aged livers, whereas oxidation of fatty acids is diminished, which leads to lipid accumulation. Insulin resistance in older people only complicates these changes, since it increases the inflow of free fatty acids and does not permit the lipid-regulation pathways to dispose of all these excessive circulating lipids. The mitochondrial dysfunction associated with aging livers may further trigger the metabolic changes in the oxidation of fatty acids, resulting in increased inflammation and oxidative stress (Q. Li et al., 2022)(H. Li et al., 2023).

2.5 Biochemical liver

The main organ involved in the metabolism of fat, protein, and carbohydrates is the liver. During the metabolism of carbohydrates, the liver controls blood sugar levels through the processes of gluconeogenesis and glycogenolysis. During fasting, glucose is produced from glycogen through a process known as glycogenolysis. On

the other hand, prolonged fasting and high exercise trigger gluconeogenesis, or the synthesis of glucose from non-carbohydrate sources (Boyer et.al., 2012).

The synthesis of plasma proteins, including the most crucial ones, albumin and clotting factors, which guarantee the preservation of blood coagulation and oncotic pressure, is a part of protein metabolism in the liver. Ammonia is created when amino acids are further demined, and this is a highly important part of hepatic activity linked to protein metabolism (Boyer et.al., 2012).

As part of lipid metabolism, the liver produces and breaks down fatty acids and triglycerides. Moreover, it is the main location of cholesterol production, which is subsequently transformed into bile acids (Boyer et.al., 2012).

Liver enzymes are essential to these metabolic processes in their entirety. Two important enzymes that play a role in amino acid metabolism are aspartate aminotransferase and alanine aminotransferase. Elevations of these enzymes in blood may occur when liver impairment is present. Any liver ailment or bile duct blockage may also result in an increase in gamma-glutamyl transferase. An enzyme involved in the bile ducts' operation; alkaline phosphatase is elevated in cholestasis patients (Boyer et.al., 2012).

Chapter III

Methods and Materials

3.1. Overview

In this natural aging paradigm, rats who are 16 months of age or older are referred to as elderly. In addition, 4-weeks rat are similar to the human age of 2 and 3 years. Furthermore, 10-weeks age rat is equivalent to the human age between 15 and 20 years.

In this research, 18 male different age Albino Wistar rats in total with an average weight of 235-740 grams were provided from the Near East University Experimental Animals Research Center (DEHAM) (Approved by the Ethics Committee No: 2024/175). All rats will be kept in conventional cages at DEHAM, with no limits on water or feed, and the same environmental and nutritional parameters (22±10 C). Regarding the age, rats were divided into three groups, each with six rats, at the start of the experiment. The groups were as follows:

- Prepuberty age Group (n=6): this group consisted of rats aged between four weeks and less than ten weeks starting from 14th day of February 2024.
- Young age Group (n=6): this group consisted of rats aged of ten weeks as January 2024.
- Adult age Group (n=6): this group consisted of rat aged of 18months starting from July 2022.

The body weight of animals was recorded at the beginning and end of the experiment. At the end of their experiment, all the rats were anesthetized with ketamine/xylazine and operated on. Their liver was quickly dissected and fixed in 10% formaldehyde solution for histological preparations. Dehydrated tissues in certain graded alcohols were cleared and embedded in paraffin. Thin sections (4-5 μm) were prepared using the paraffin blocks and stained with Hematoxylin-Eosin (HE), Masson's Trichrome, and for microscopic examination. Blood samples were collected in clot activator tubes and also taken for analysis to a biochemistry laboratory. Liver samples were also collected in Eppendorf tubes for further examination in a genetic lab using PCR. Finally, the results achieved were analyzed using Graphpad.

3.2. Hematoxylin and Eosin staining.

1. The liver tissue was kept in an etuv oven machine at 60°C for 12 hours.
2. After the oven, the liver tissue was placed in xylene and kept there for 10-15 minutes.
3. The liver tissue was then kept in xylene at room temperature for an additional minute.
4. After completing xylene process, the liver tissue was placed in 100% alcohol for 1 minutes.
5. Immediately after 100% alcohol, the liver tissue was placed in 80% alcohol for 1minute, then 70% alcohol for 1 minute.
6. The liver tissue was immediately washed in distilled water for 1 minute following alcohol treatment.
7. After removing alcohol from the tissue, we placed in hematoxylin for 4 minutes.
8. After hematoxylin, the tissue was washed twice and then placed in distilled water for 10 minutes.
9. The tissue was placed in Eosin stain for 1 minutes and immediately washed.
10. The washed tissue was then and we put in again in alcohol, 80%, 90% and 100% for 1 minutes each.
11. After drying, the tissue was cleared in xylene at room temperature and mounted with Entellan with coverslip.

3.3. Masson Trichrome (BIO-OPTICA 04-010802- MILAN/ITALY) Staining Method:

1. The tissues were kept in hot xylene for 1 minute.

2. Then, the tissues were kept in xylene at room temperature for 1 minute.

3. After completing the xylene process, the tissues were placed in 100% alcohol for 1 minute.

4. The tissues were kept in 80% alcohol for 1 minute.

5. While in alcohol, 30 drops of Hematoxylin A and 30 drops of Hematoxylin B were mixed.

6. The preparations removed from the alcohol were placed on the staining containers.

7. The prepared Hematoxylin A and Hematoxylin B mixture was dripped onto the preparations. They were left for 10 minutes and then washed with distilled water.

8. The preparations were left to dry for 20 minutes.

9. After dripping 1% Picric acid onto the tissues, they were left for 10 minutes.

10. Then the tissues were immediately washed with distilled water for 1 minute.

11. After removing the tissues from the distilled water, Fuchsin was dripped and left for 4 minutes.

12. Phosphomolybdic Acid was dripped onto the tissues and left for 10 minutes.

13. Immediately after Phosphomolybdic Acid, the tissues were washed with distilled water for 1 minute.

14. After washing, Masson Aniline was dripped onto the tissues and left for 5 minutes.

15. Then the tissues were washed with distilled water for 1 minute, dried, cleared in xylene, and mounted with Entellan.

3.4 periodic acid-Schiff (PAS) staining method for histological section.

1. The liver tissue was kept in an etuv oven machine at 60°C for 12 hours.
2. After the oven, the liver tissue was placed in xylene and kept there for 10-15 minutes.
3. Bring section to distilled water.
4. Put on the section 10 drops of reagent A: leave to act 10 minutes.
5. Wash in distilled water.
6. Put on the section 10 drops of reagent B: leave to act 20 minutes.
7. Wash in distilled water.
8. Put on the section 10 drops of reagent C: leave to act 2 minutes.

9. Drain the slides without washing add 10 drops of reagent D: leave to act 2 minutes. Rinse in distilled water.
10. Put on the section 10 drops of reagent E and wait 3 minutes.
11. Wash in running tap water for 5 minutes
12. Dehydrate through ascending alcohols. Clear in xylene and mount.

3.5. RNA ISOLATION

1. Tissues: Homogenize tissue samples in 1 ml of TRIZOL reagent per 50 to 100 mg of tissue.
2. Add 500 ml of TRIZOL and 100 ml chloroform Reagent. Cap sample tubes securely.
3. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes.
4. Centrifuge the samples at no more than 14,000 x g for 15 minutes at 2 to 8°C.
5. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase.
6. RNA remains exclusively in the aqueous phase.
7. Transfer upper aqueous phase carefully without disturbing the interphase into a fresh tube.
8. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 250 µl of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization.
9. Incubate samples at 15 to 30°C for 10 minutes.
10. Centrifuge at no more than 10,000 x g for 10 minutes at 2 to 4°C.
11. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
12. Remove the supernatant completely.
13. Wash the RNA pellet once with 75% ethanol, adding at least 500 µl of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization.
14. Mix the samples by vortex and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8°C.
15. Repeat the above washing procedure once. Remove all leftover ethanol.

16. Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under vacuum.
17. Add 25 µl µl DNase RNase-free water to elute RNA.

3.6. RNA converts cDNA

Sample preparation

In all, 19 samples were prepared, including one extra to cover possible residue or wastage during micro pipetting. The following reagents and their quantities were used for each sample:

1. Buffer: 4 µl of buffer for one sample, totaling 76 µl for all 19.
2. dNTP (Deoxynucleotide Triphosphates): 1 µl per sample.
3. Supplied with Dt Primer: 1 µl for all samples, a total of 19 µl for 19 samples.
4. RT (Reverse Transcriptase) Enzyme: 1 µl per sample, total for 19 µl.
5. Distilled Water (D H_2O): 3 µl per sample, for a total of 57 µl.
6. RNA Addition
7. To each reaction of the prepared samples, 10 µl of RNA was added, giving a final volume of 20 µl for the cDNA synthesis.
8. Shake it gently, mix the reaction, and use the centrifuge shaker.
9. Perform the cDNA synthesis by incubation for 15 min at 50-55°C.
10. Stop the reaction by heating to 85 °C, incubating for 5 minutes, and storing at −20 °C.

Procedure.

- Reagent Mixing: The measured volume of buffer, dNTP, Dt primer, RT enzyme, and distilled water was mixed for 19 sample tubes.
- RNA Addition: 10 µl of RNA was added to each tube.
- cDNA Synthesis: The mixture was then incubated for cDNA synthesis from RNA.
- Oligo dT: This is used for RNA isolated from eukaryotes because of their poly-A tails.
- Random hexamer: used for RNA isolated from prokaryotes since they do not have poly-A tails.

3.7. protocol for a quantitative PCR reaction using a SYBR Green master mix.

Separate Components (1X and 18X)

Component	Volume for 1X (µL)	Volume for 18X (µL)
SYBR Green master mix (SYBR)	5	90
Forward Primer (F)	0.5	9
Reverse Primer (R)	0.5	9
Distilled Water (dH_2O)	1	18
Total (without cDNA	7	126

Master mix preparation steps

Step	Description
Prepare Master Mix	Combine SYBR, Forward Primer, Reverse Primer, and dH_2O. Total volume for 18 reactions: 126 µL.
Aliquot Master Mix	Distribute 7 µL of the master mix into each of the 18 qPCR reaction tubes.
Add cDNA	Add 3 µL of cDNA to each reaction tube.

cDNA Dilution Ratio	Volume (µL)
cDNA	54
Distilled Water (dH_2O)	216
Total	270

c-DNA Dilution for 18 Reactions

Calculation for Dilution

- For 1 reaction
  - 1 µL cDNA + 4 µL dH_2O
- For 18 reactions
  - 3µL×18=54µL of cDNA
  - 54µL×4=216µL of distilled water

Final Volume per Reaction.

Component	Volume per Reaction (µL)
Master Mix	7
cDNA	3
Total	10

The next protocol integrates all the steps in detail for preparation of qPCR reactions, cDNA dilutions, and the PCR program, followed by another 120-min waiting at 4°C within the PCR machine.

3.8. Biochemical method:

The enzyme activities of AST (Aspartate aminotransferase), ALT (Alanine aminotransferase) and ALP (Alkaline phosphatase), as well as the levels of cholesterol and triglycerides, were measured using enzymatic methods on the Abbot architect c400 system (Abbot Laboratories, IL, USA).

CHAPTER IV

Results and Discussion

4.1 Statistical analysis.

The data resulting from the subjects' body weights before and after the experiment, as well as their testicular weights, were statistically evaluated using the GraphPad Prism 8.3.1 program. Data were compared using the GraphPad Prism 8.3.1 program and Two-way ANOVA. A significance threshold of $p \leq 0.05$ was reached, and a standard error of ± was allowed. Table 4 presents a comparison of the groups' mean ± SD and p values.

Types of rats	Number	Body weight (Mean ± SD)	Liver weight (Mean ± SD)
4 weeks group	6	285 ± 27.96	3.693 ± 0.5747
10 weeks group	6	332± 27.30	3.785 ± 0.7838
18 months group	6	631 ± 78.31	3.594 ± 6.6655

Table 4.1. Comparison of body and liver weights of different age groups

The table gives the body and liver weights of rats at four weeks, ten weeks, and eighteen months; this study involves six rats in each group compared on characteristic traits. Weights have been presented as means along with SDs standard deviations; statistically, research proves that there is a significant change with age in body weight under development where this considerable difference can be viewed by

the very high significance level that was 4 weeks group p<0.0001, 10 weeks 0.0292-, and 18-months P<0.0001 between different ages for body weight. The probability that observed differences are due to chance is expressed by "p," the symbol for a p-value. A p-value less than 0.05 is generally considered significant; values below 0.0001 show good evidence against the null hypothesis.

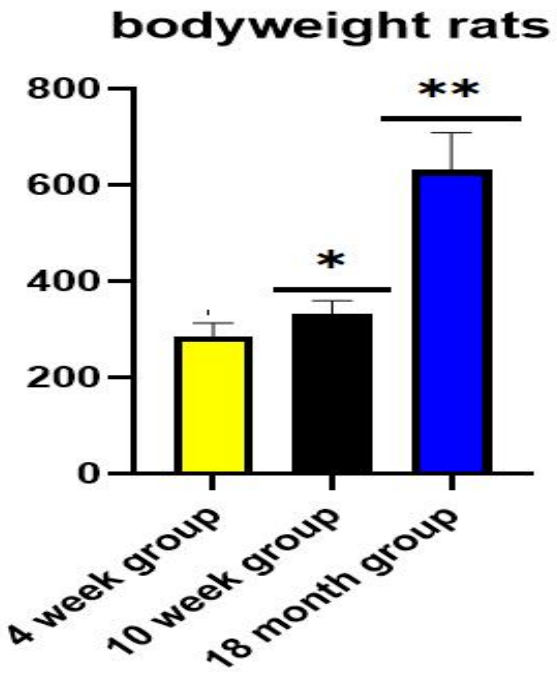


Figure 4.1.1. body weight development in rats across different age groups.

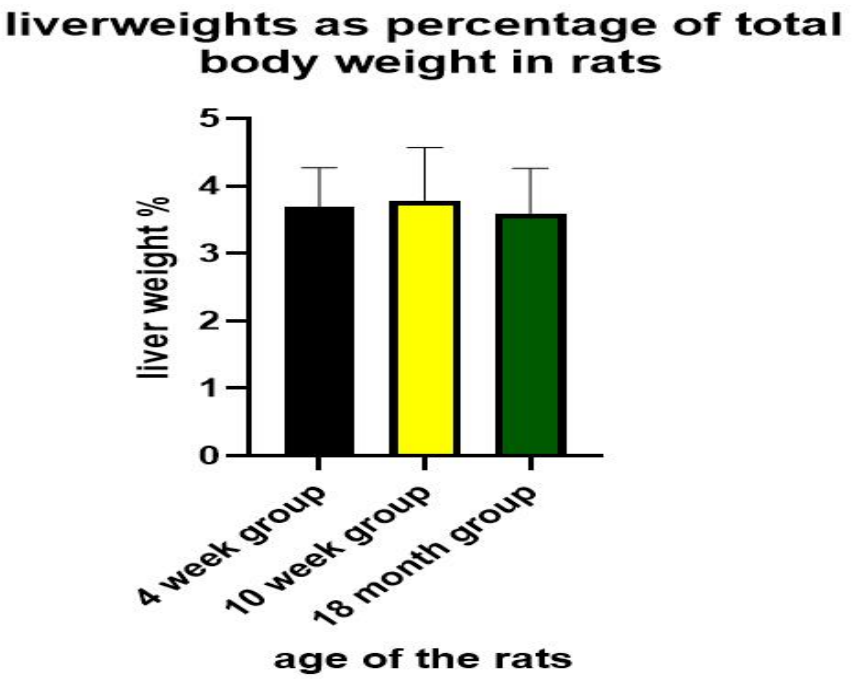


Figure 4.1.2. liver weight as percentage of total body weight across different age groups of rats.

4.2: Biochemistry analysis

Types Of rat groups	Number	CHOLESTROL (Mean ± SD)	TRIGLYCERIDES (Mean ± SD)	AST (Mean ± SD)	ALT (Mean ± SD)	ALP (Mean ± SD)
4 week group	6	59.17±7.731	77.83±13.15	95.67 ±11.4 1	51.83 ±5.56 5	204.7± 32.20
10 week group	6	70.17±7.653	68.17±45.80	111.8 ±21.9 2	51.17 ±23.3 0	283.5± 42.93
18 months group	6	113.3±15.36	121±45	136.5 ±28.8 9	85±33 .16	410.2± 90.15

Table 4.2. comparison of lipid and liver enzyme levels across different age groups of rats.

This table presents the measurement results of rat biochemical parameters in three age groups. The results are represented as mean values ± SD. The numerical values that denote the degree of evidence opposing the null hypothesis are: cholesterol, p = <0.0001; triglycerides, p = <0.0001; AST, p = <0.0001; ALT, p = <0.0001; ALP, p = <0.0001. this would be a statistically significant difference, probably showing actual trends with age in such parameters.

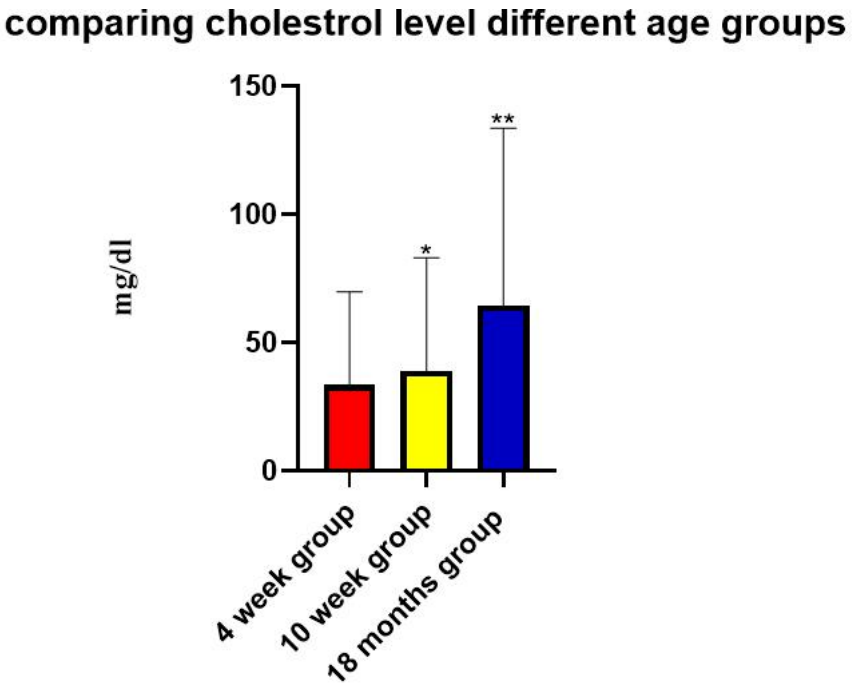


Figure 4.2.1. Biochemical profile of comparing cholesterol level different age group.

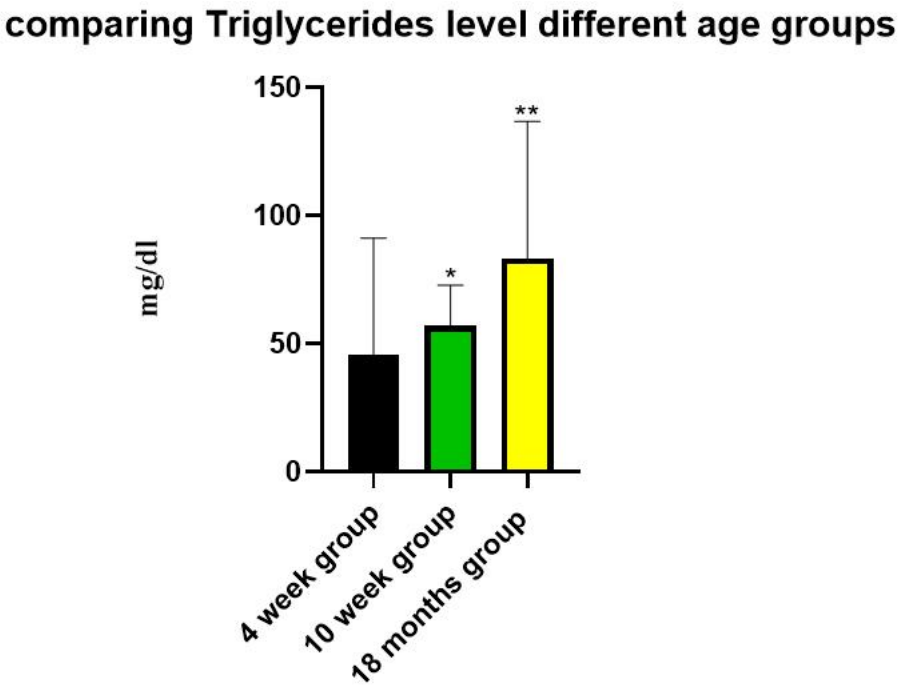


Figure 4.2.2. Biochemical profile of comparing triglycerides level different age group.

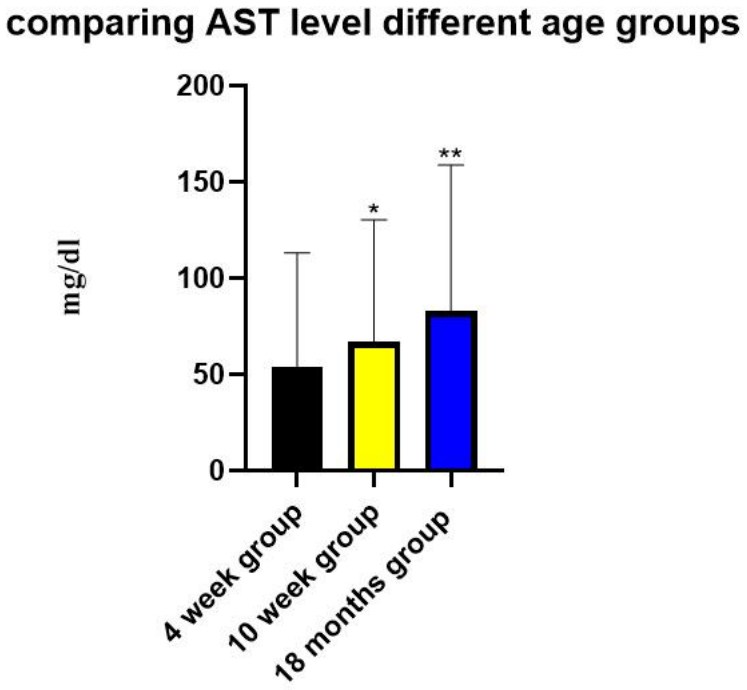


Figure 4.2.3. Biochemical profile of comparing AST level different age group.

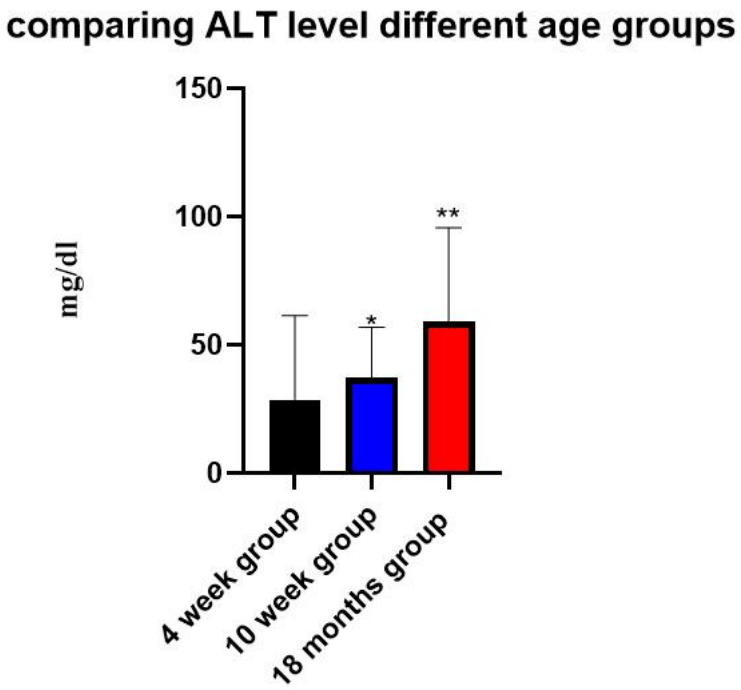


Figure 4.2.4. Biochemical profile of comparing ALT level different age group.

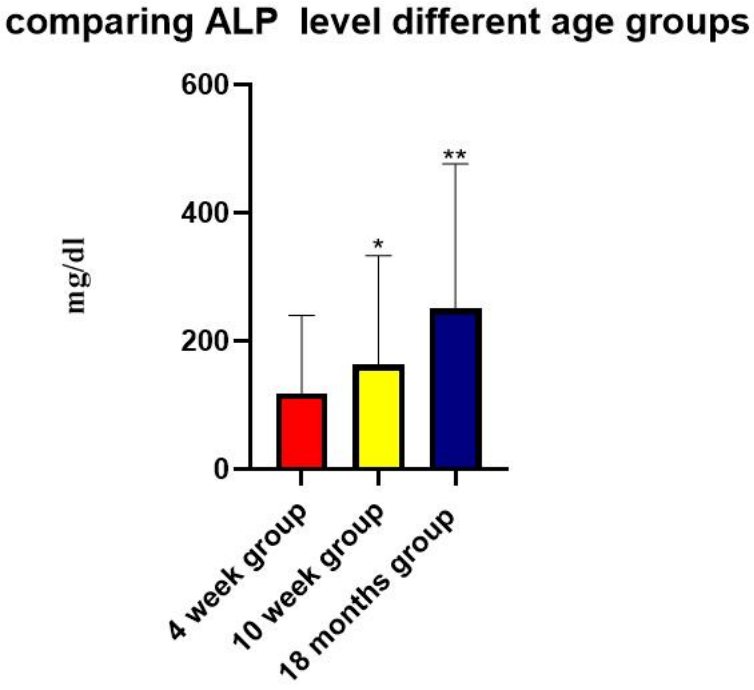


Figure 4.2.5. Biochemical profile of comparing ALP level different age group.

4.3 Double nucleated hepatocytes in rats.

(Binuclear hepatocytes were counted in 10 different places using an x40 objective magnification in the tissue section of each individual.)

Types of rat's groups (n=6)	Mean ± SD
4-week group	1.983±1.112
10-week group	4.967±2.178
18 months group	8.433±3.734

Table 4.3. Age-related changes in double nucleated hepatocytes

This table summarizes the increase in double nucleated hepatocytes among rats aged 4 weeks, 10 weeks, and 18 months examined across 10 areas. Data are expressed as mean ± SD. The symbol "P" stands for p-value, a statistical measure customarily used to determine the significance of results obtained from a hypothesis test. A p-value <0.0001, reported here, means a statistically significant increase in the frequency of double nucleated hepatocytes with age, showing prominent changes in liver cell morphology over time.

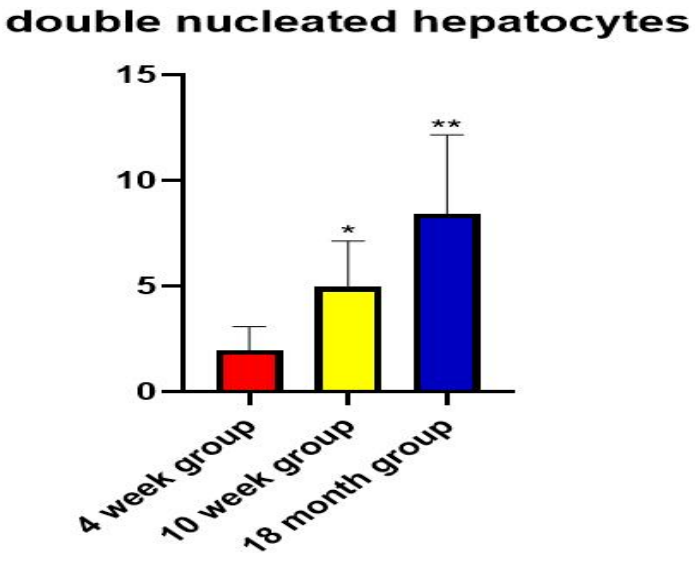


Figure 4.3.1. Age-related increase in double nucleated hepatocytes in rat liver tissues.

4.4: Histological scoring.

(Scoring was done on the sections of the subjects in each group with an objective magnification of x 40. Five areas in each section were scanned for enlargement of Sinusoidal Dilation Congestion, Mononuclear cells, Vacuolization Lipid Increase, fibrosis and bile duct proliferation).

types of rat group (n=6)	Sinusoidal Dilation Congestion (Mean ± SD)	Mononuclear cells (Mean ± SD)	Vacuolization Lipid Increase (Mean ± SD)	Fibrosis	Bile Duct Proliferation (Mean ± SD)
4 week group	0.2000± 0.406	0.06667 ±0.2537	0.000±0.000	0.000± 0.000	0.000±0.000
10 week group	0.5667±0. 5683	0.4667± 0.5713	0.2000±0.484 2	0.0333 3±0.18 26	0.1000±0.30 51
18 months group	0.9667±0. 7184	0.4667± 0.5713	0.7667±0.626 1	0.4000 ±0.498 3	0.5000±0.57 24

Table 4.4.1. Quantitative histological evaluation across different age groups of rats.

This table shows the histology scores for the liver parameters with the mean values and SD for each age group of rats. The "p-value" is an index of the statistically significant changes between these groups of rats; $p < 0.0001$ indicates highly substantial changes, thus confirming that there are age-related structural alterations in liver histology.

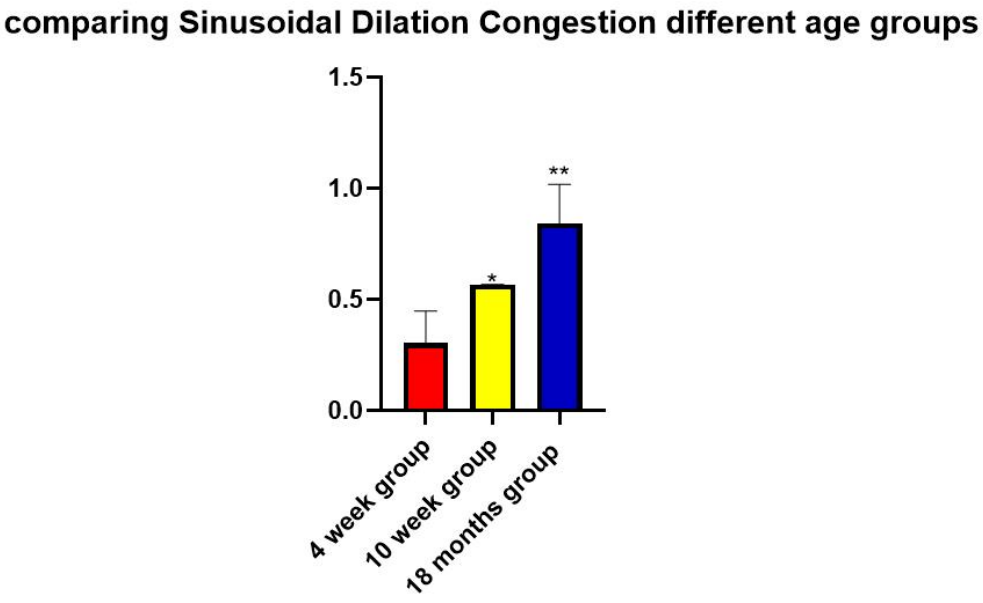


Figure 4.4.1. comparing Sinusoidal Dilation Congestion across different age group.

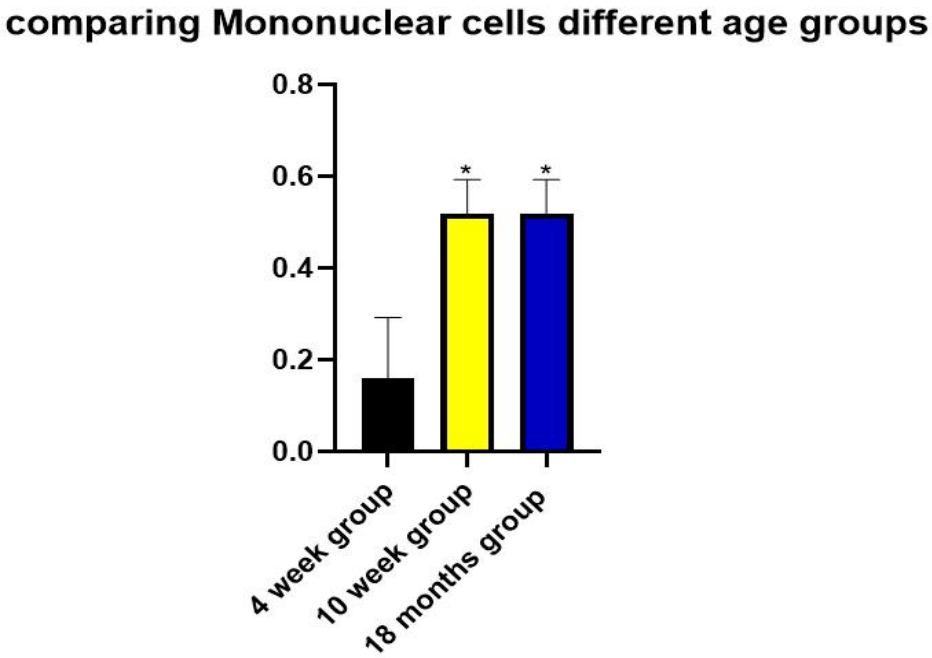


Figure 4.4.2. comparing Mononuclear cells across different age group.

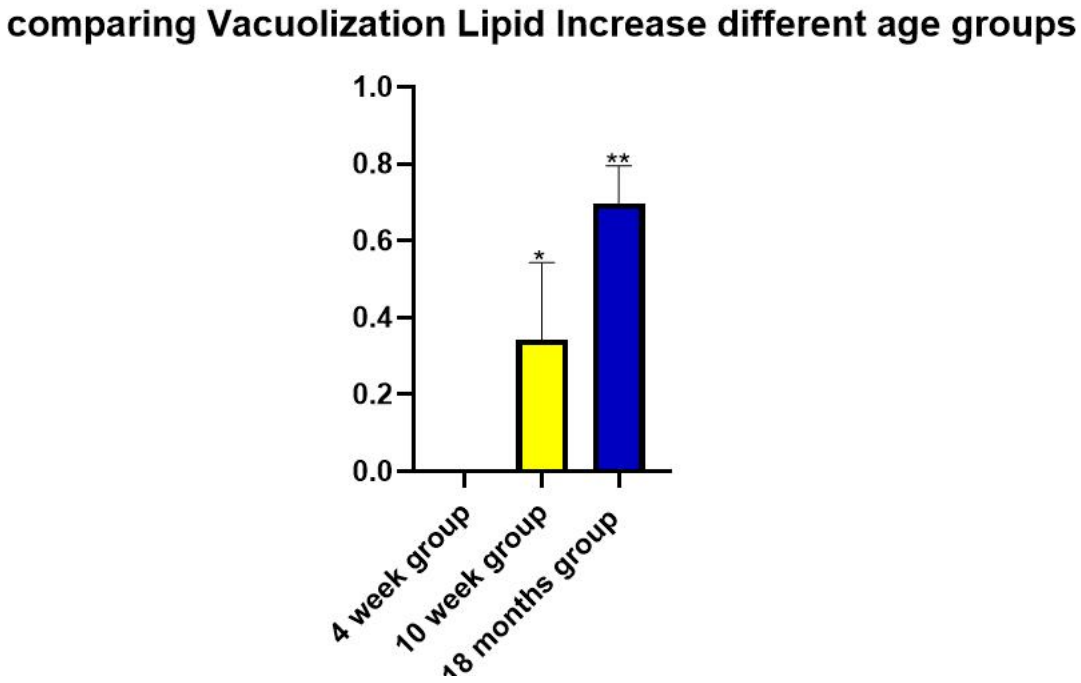


Figure 4.4.3 comparing Vacuolization Lipid Increase across different age group.

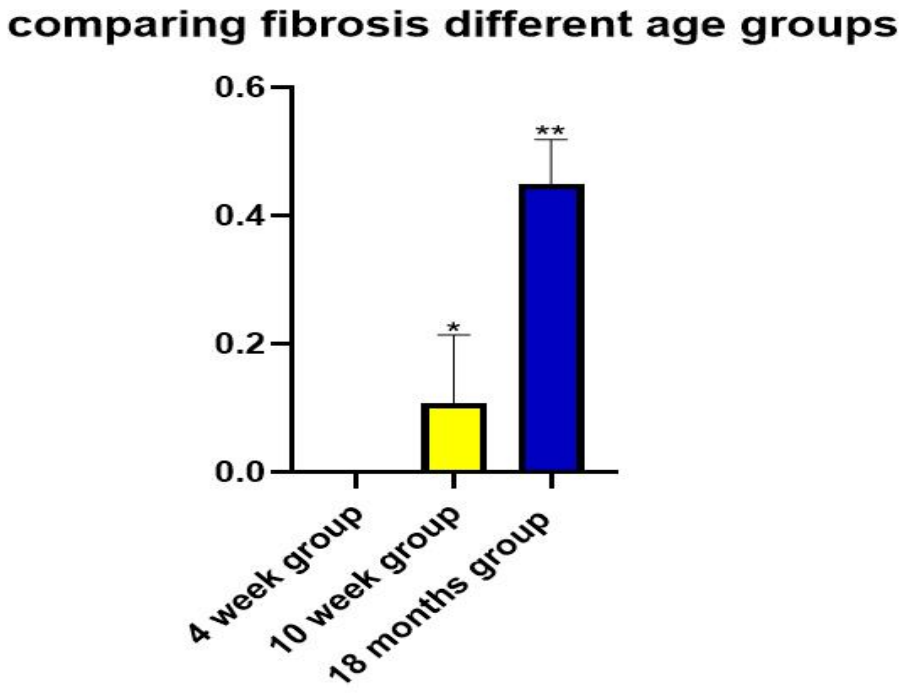


Figure 4.4.4. comparing fibrosis across different age groups.

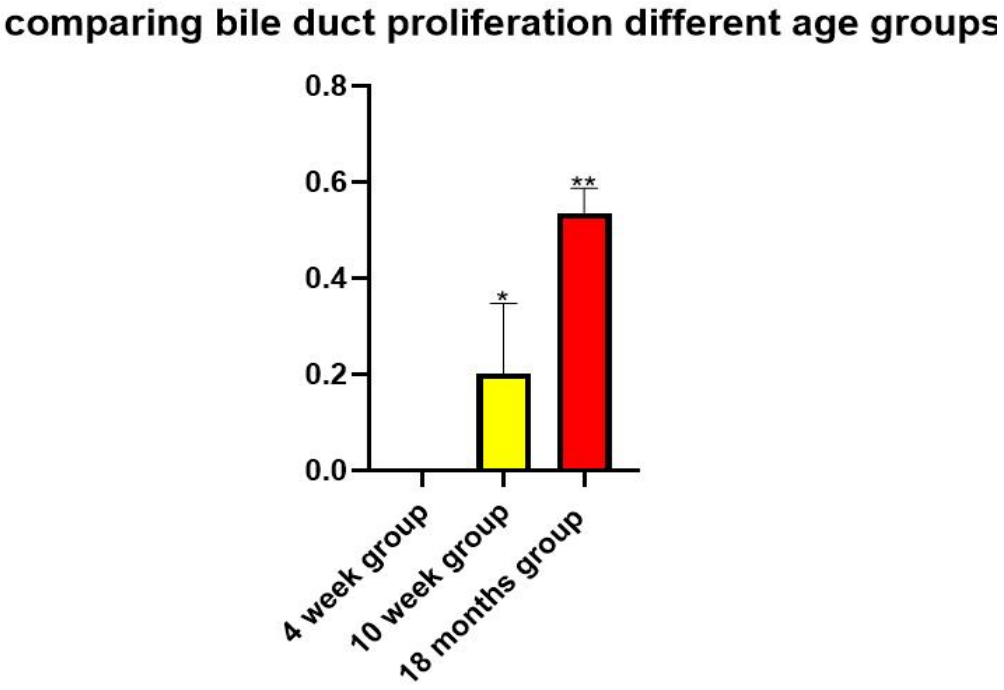


Figure 4.4.5. comparing bile duct proliferation across different age groups.

4.5. Gene expression analysis.

Quantitative PCR is an invaluable investigative tool in molecular biology for quantifying gene expression, which is very important for the understanding of developmental biology, mechanisms of disease, and genetic regulation. This study extends the usual analysis by investigating changes in the expression of some essential genes Interleukin-6(il-6), Mammalian Target of Rapamycin (MTOR), Caspase 3, and Nuclear Factor Kappa B(NFKB) across three rat age groups: prepuberty (4-week-old rats) young (10-week-old rats), and aged (18-month-old rats). The differences in expressing IL-6, MTOR, CASP3, and NFKB with regarding the Mean, Standard deviation and multiple comparisons test (P-value Significantly different) has been found out.

4.5.1 IL-6

Types of rat's groups (n=6)	Mean	Std. Deviation	multiple comparisons test (P-value Significantly different).
4 weeks	1.354	0.2432	4-week group vs. 10-week group. YES 0.0230
10 weeks	3.912	0.2857	10-week group vs. 18 months group. NO 0.1414
18 months	5.155	0.6993	4-week group vs. 18 months group YES 0.0075

Table 4.5.1.1. IL-6 levels in rats at different ages with statistical comparisons.

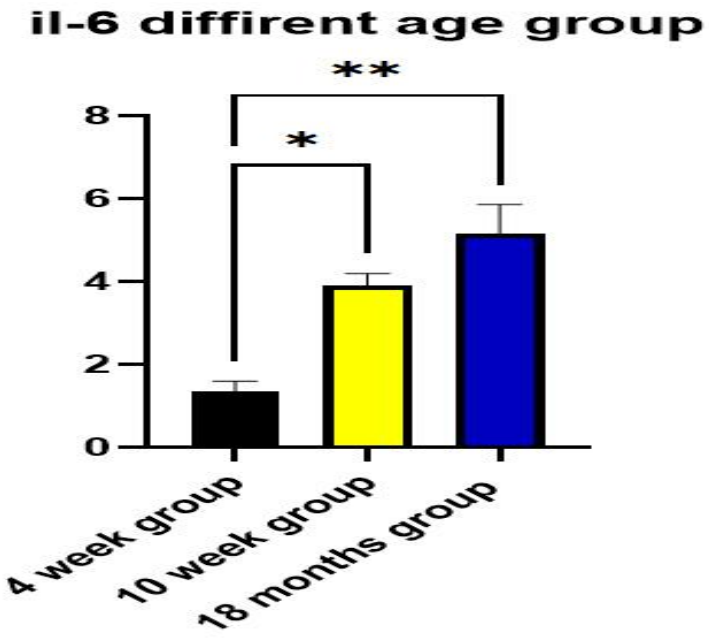


Figure 4.5.1. IL-6 levels across different Age groups in rats.

4.5.2. MTOR

Types of rat's groups (n=6)	Mean	Std. Deviation	multiple comparisons test (P-value Significantly different).
4 weeks	1.346	0.04525	4 weeks group vs. 10 weeks group. No 0.0595
10 weeks	3.830	0.9758	10 weeks group vs. 18 months group. YES 0.0366
18 months	6.823	0.5091	4 weeks group vs. 18 months group. YES 0.0067

Table 4.5.2.1. M-TOR levels in rats at different ages with statistical comparisons.

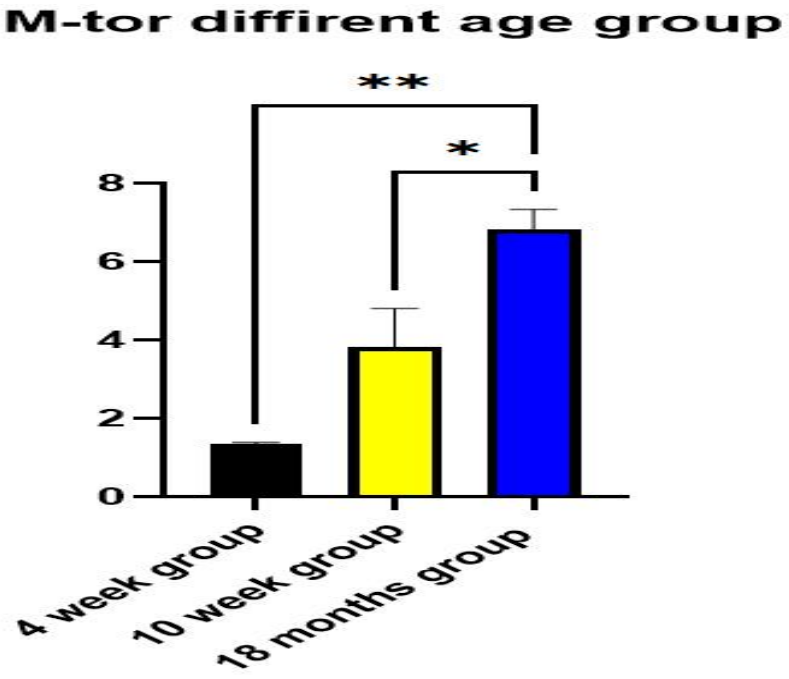


Figure 4.5.2. M-TOR levels across different Age groups in rats.

4.5.3: Caspase 3

Types of rat's groups (n=6)	Mean	Std. Deviation	multiple comparisons test (P-value Significantly different).
4 weeks	1.054	0.04172	4 weeks group vs. 10 weeks group. YES <0.0001
10 weeks	5.523	0.1541	10 weeks group vs. 18 months group. YES 0.0003
18 months	8.414	0.1273	4 weeks group vs. 18 months group. YES <0.0001

Table 4.5.3.1 Caspase 3 levels in rats at different ages with statistical comparisons.

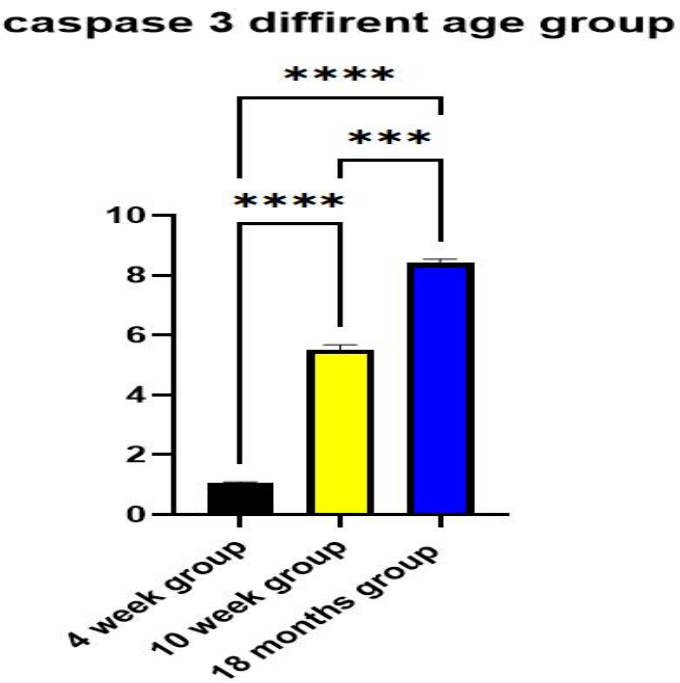


Figure 4.5.3. Caspase 3 levels across different Age groups in rats.

4.5.4: NFKB GENE

Types of rat's groups (n=6)	Mean	Std. Deviation	multiple comparisons test (P-value Significantly different).
4 weeks	1.187	0.2680	4 weeks group vs. 10 weeks group. Yes 0.0267
10 weeks	8.318	2.256	10 weeks group vs. 18 months group No 0.8679
18 months	9.019	0.5438	4 weeks group vs. 18 months group Yes 0.0206

Table 4.5.4.1. NFKB levels in rats at different ages with statistical comparisons.

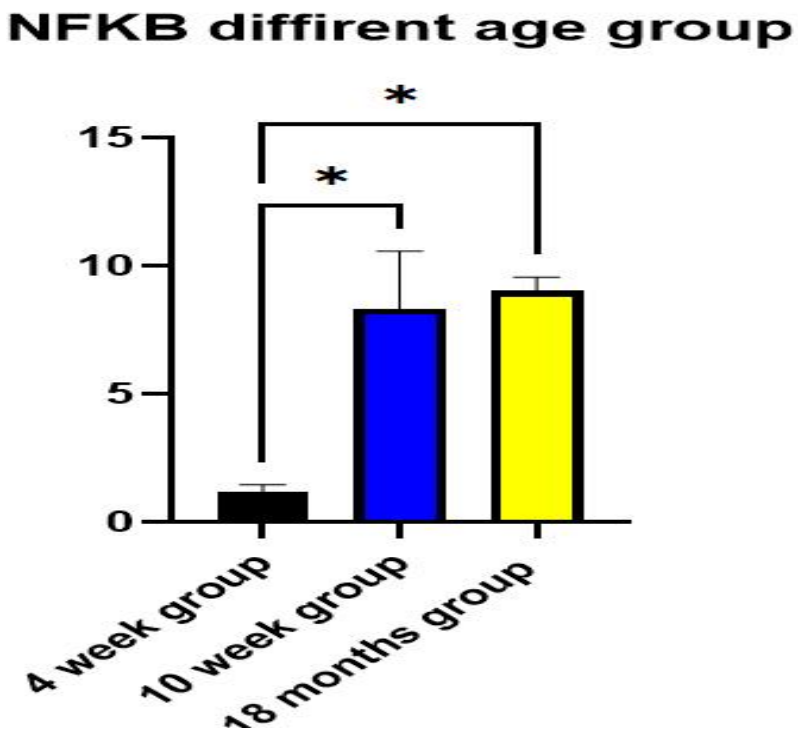


Figure 4.5.4. NFKB levels across different Age groups in rats.

4.6: Light Microscopic Evaluation

Light microscopic evaluation in tissues was made using x10 and x40 objective magnification.

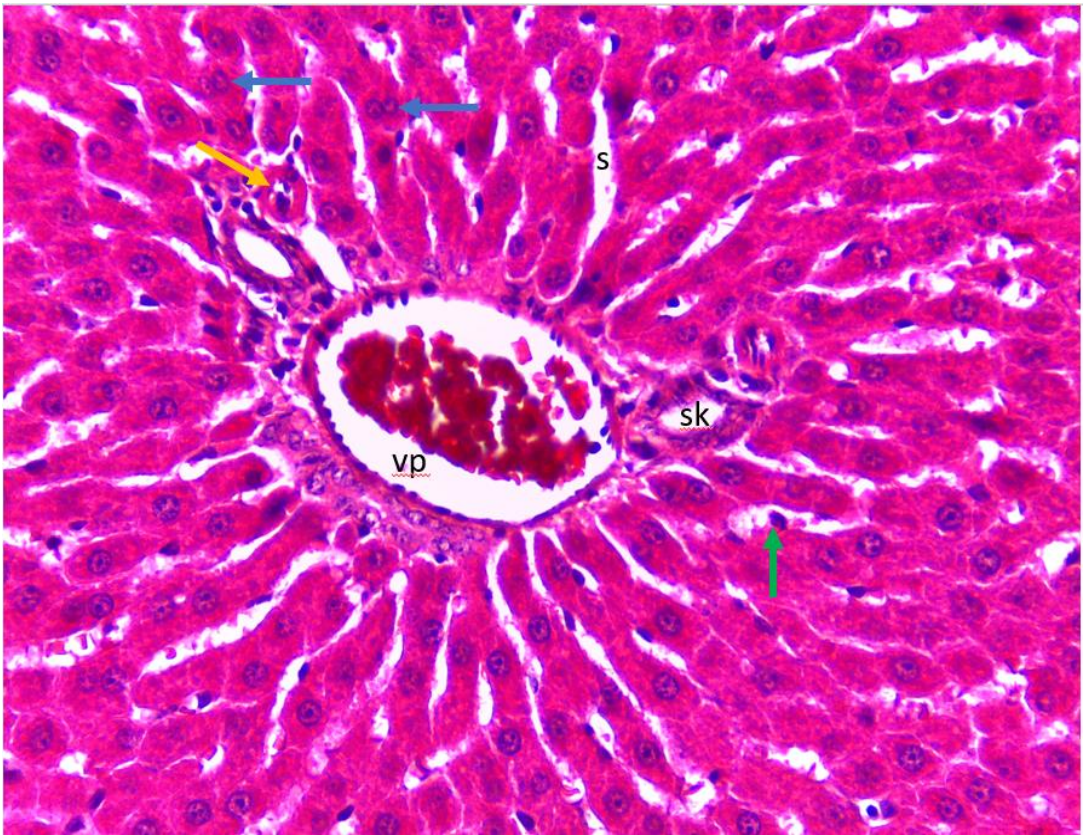


Figure 4.6.1. The liver tissue of a 4-week-old rat shows hepatocytes (h), sinusoids (s), binucleated hepatocytes (blue arrow), and Kupffer cells (green arrow) in the portal area and adjacent locations. Hepatica branch (yellow arrow), bile duct (sk), and vena porta branch (vp) x40 Hematoxylin and Eosin stain

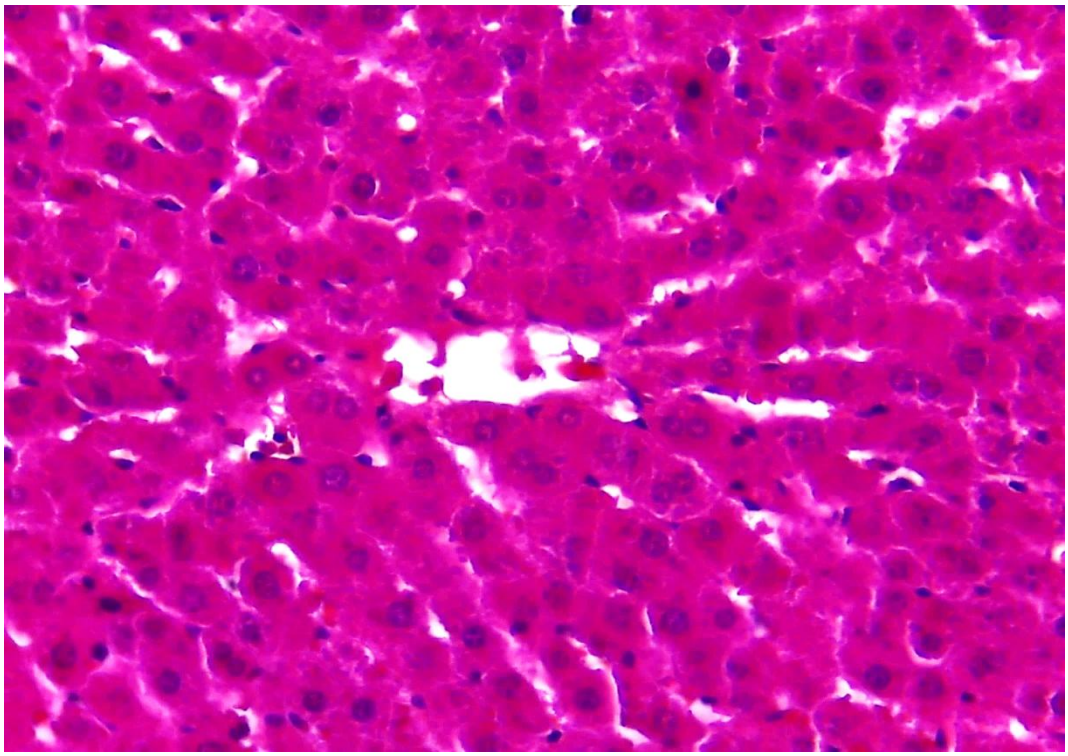


Figure 4.6.2. Hematoxylin and eosin staining, x40. Centralis (VS) and radially extending hepatocytes and sinusoids are visible in the liver tissue of a 4-week-old rat.

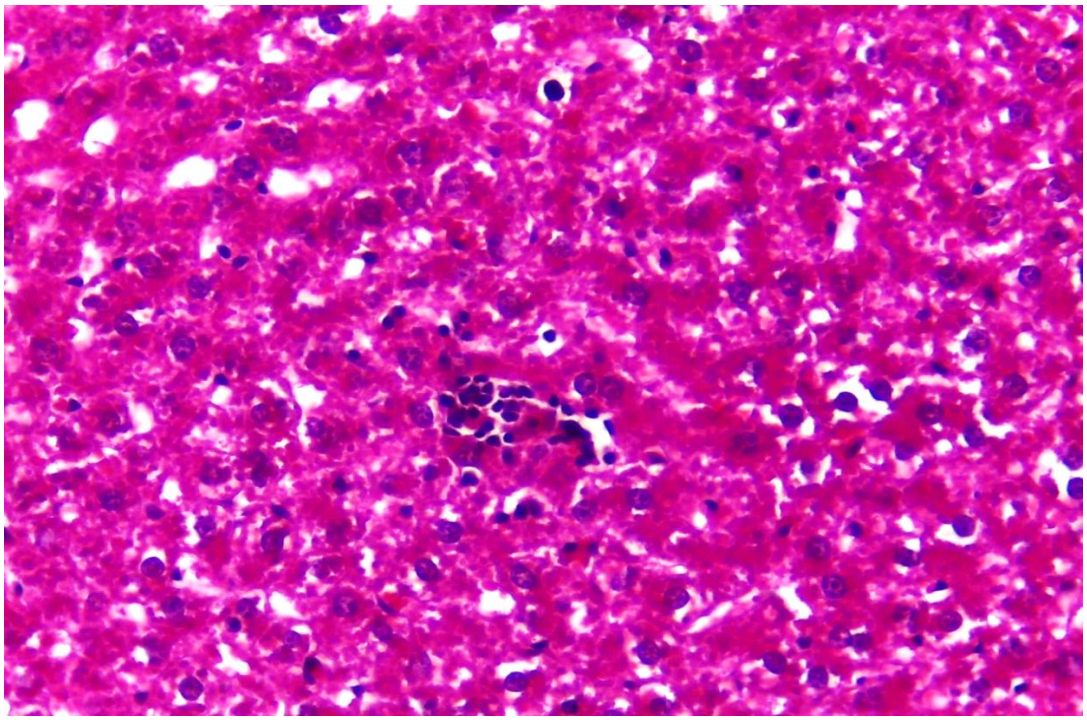


Figure 4.6.3. Uncommon inflammatory cell aggregates are visible in the 4-week-old rat group (marked area). X40 hematoxylin and eosin staining.

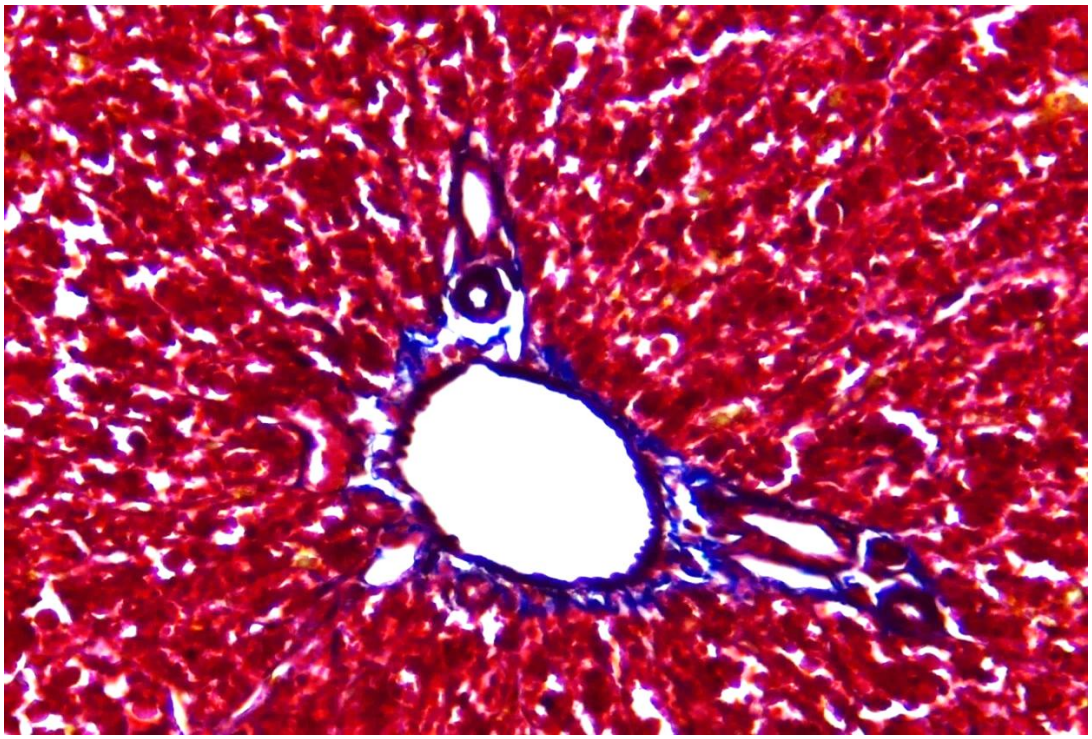


Figure 4.6.4. Collagen fibrils-stained blue are visible in the portal area of the 4-week-old rat group. Staining Masson trichrome x40

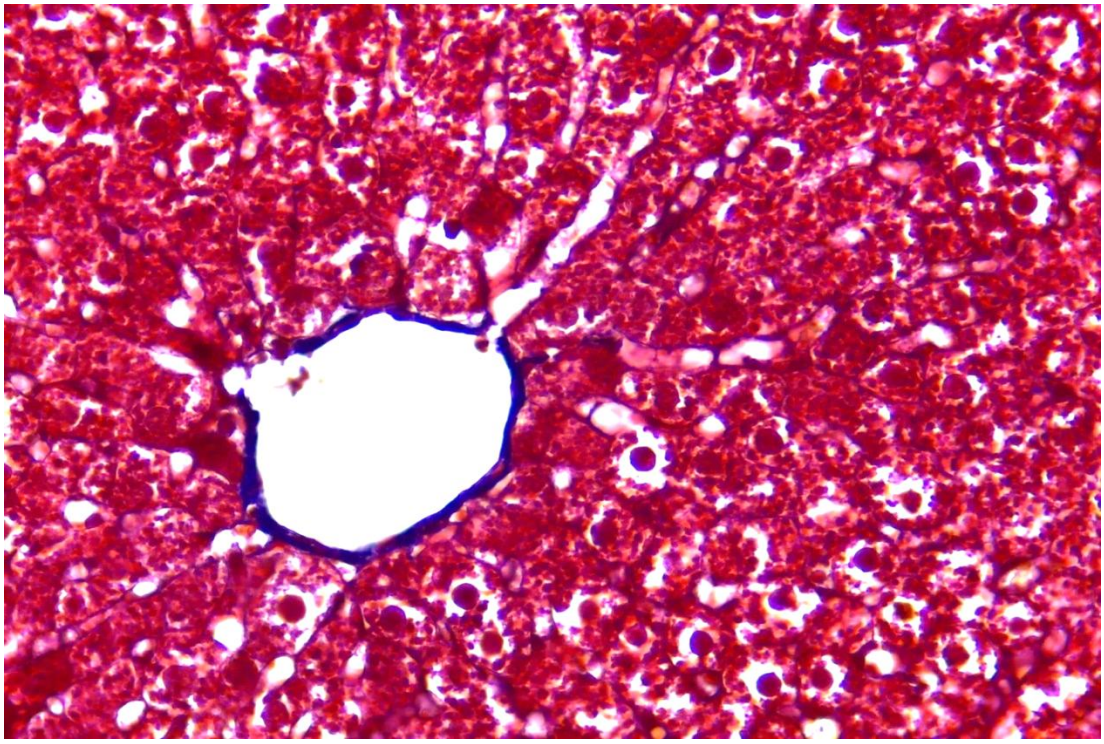


Figure 4.6.5. A group of 4-week-old rats Around the V. Centralis, collagen fibers are visible. x40 Masson trichrome stain.

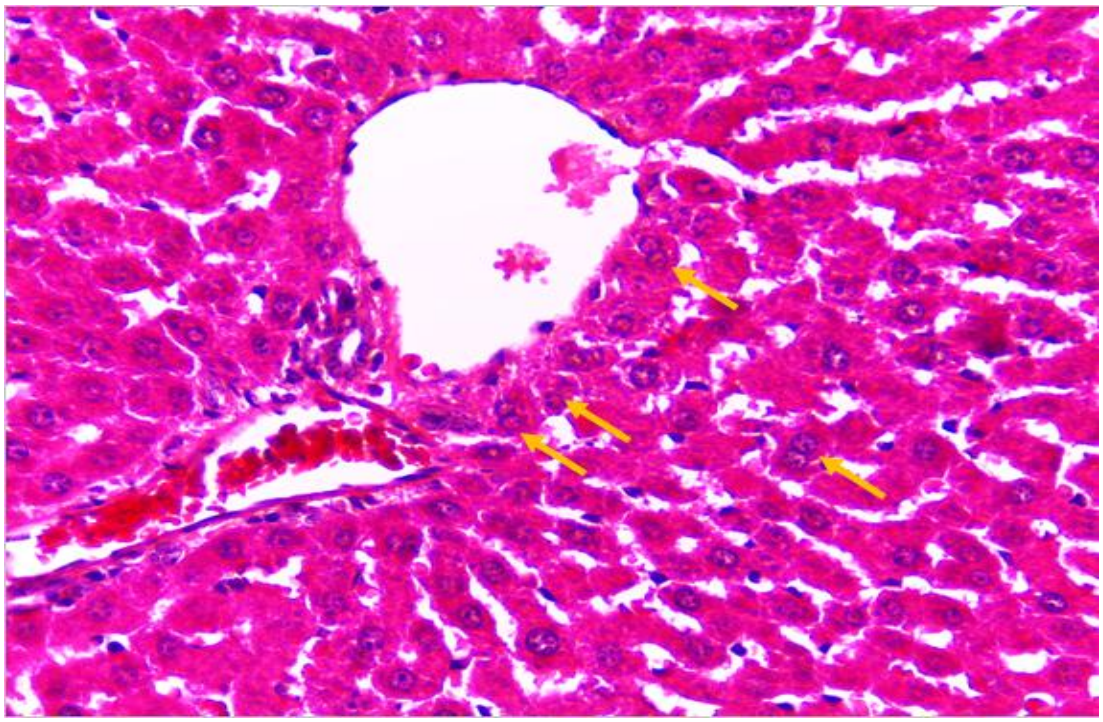


Figure 4.6.6. Liver tissue from 10-week-old rats shows hepatocyte cell cords and a large number of binucleated hepatocytes (yellow arrows): x40 Hematoxylin and Eosin staining.

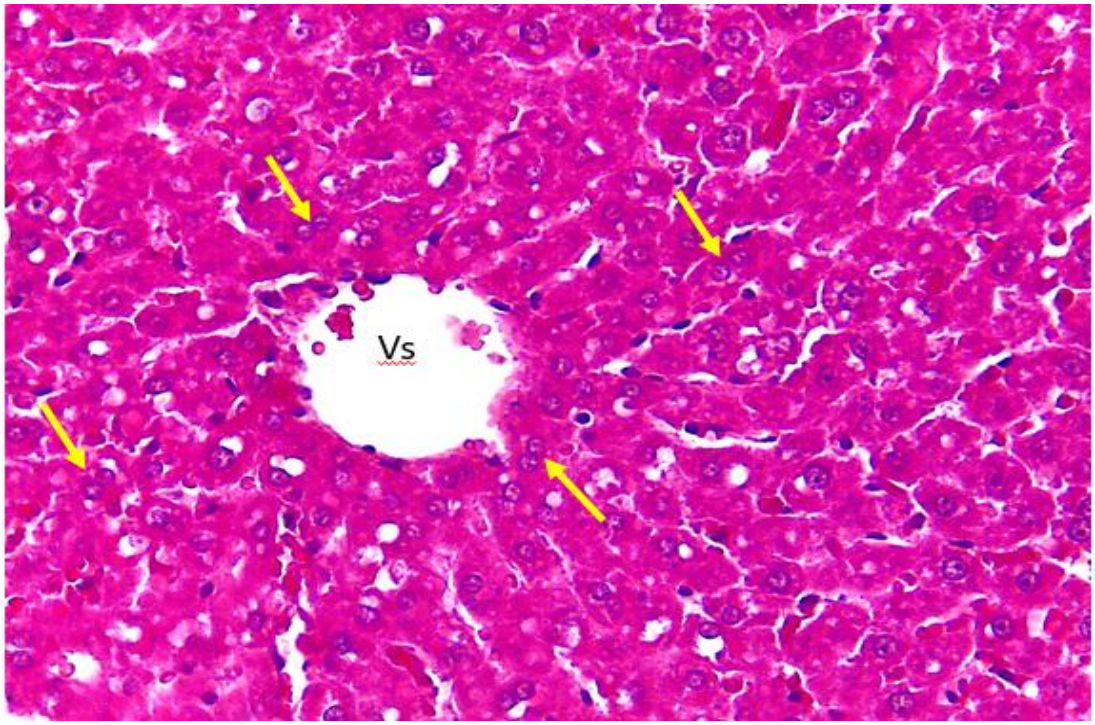


Figure 4.6.7. Around the central vein are binuclear hepatocytes (yellow arrows) from the 10-week group. x40 Hematoxylin and Eosin staining.

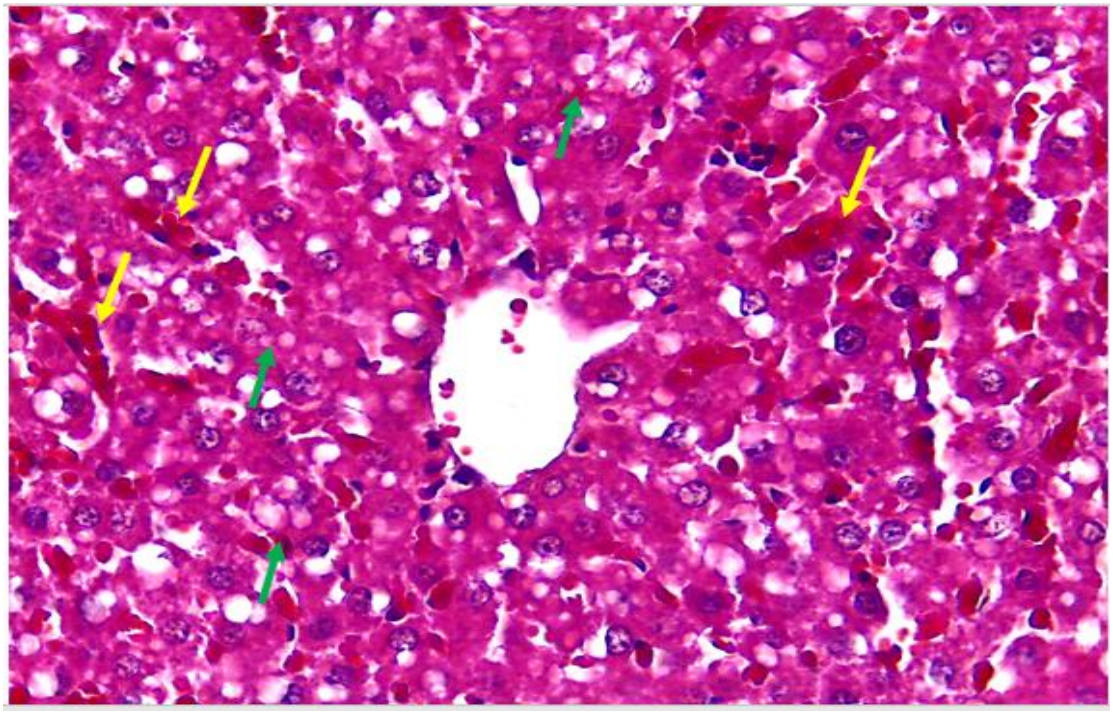


Figure 4.6.8. In the 10-week group, the sinusoids around the vena centralis show signs of vacuolization (green arrows) and congestion (yellow arrows). x40 Hematoxylin and Eosin staining.

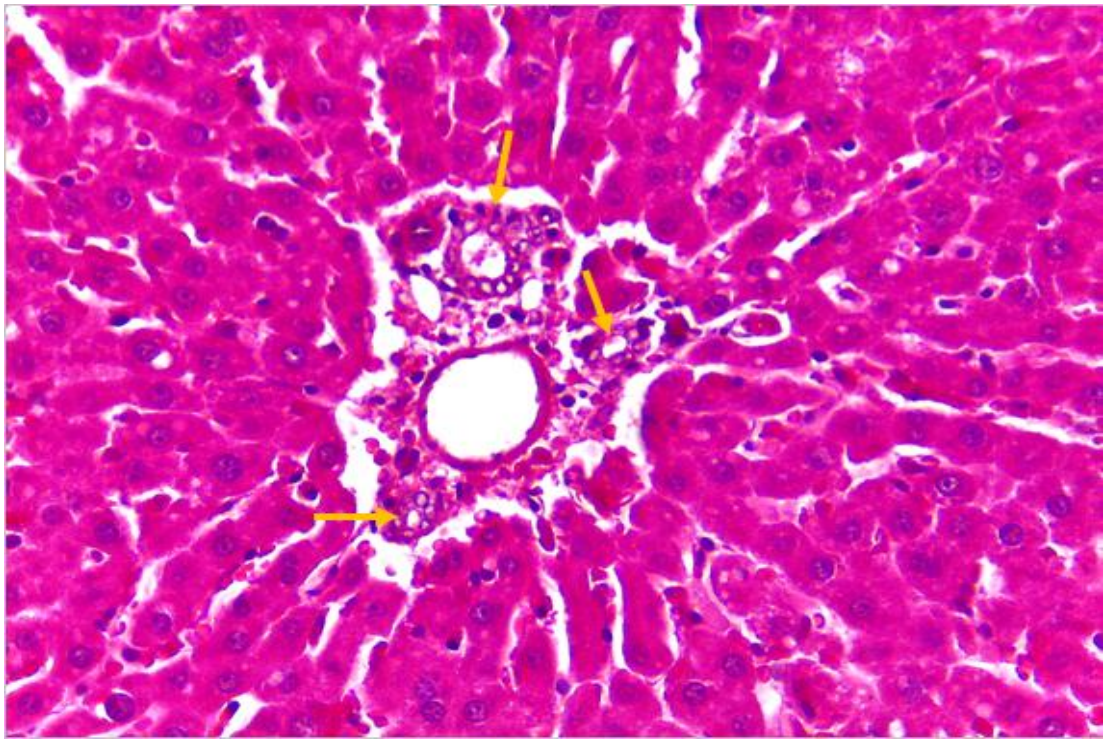


Figure 4.6.9. The portal area has modest bile duct hyperplasia (yellow arrows). x40 Hematoxylin and Eosin staining.

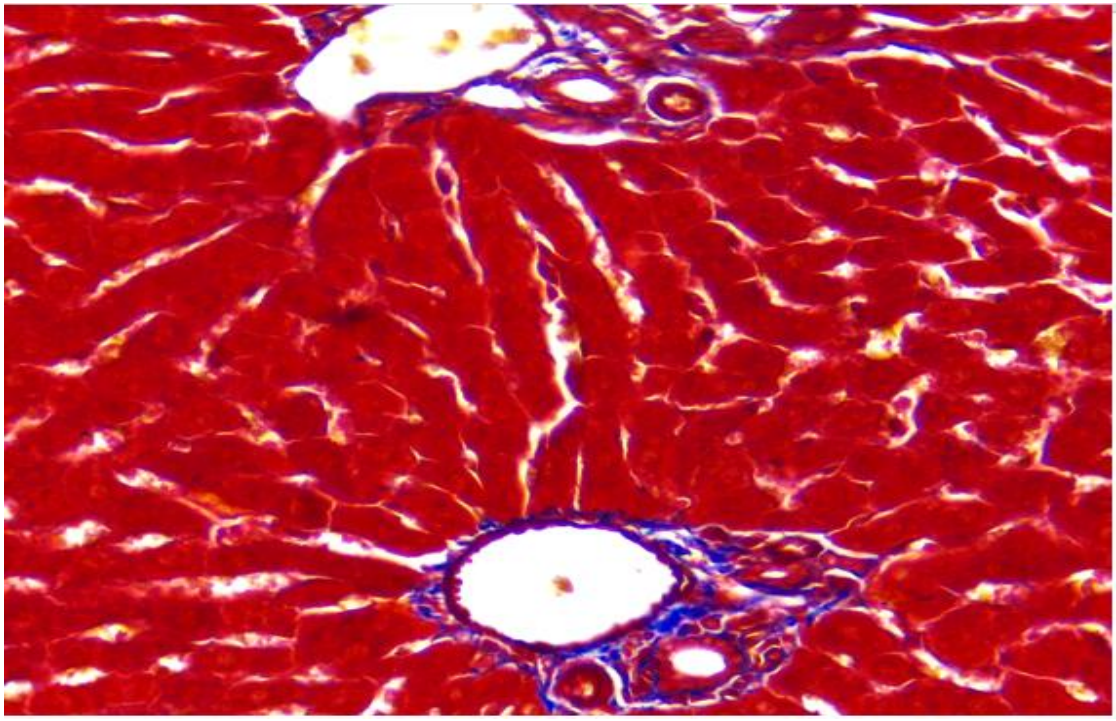


Figure 4.6.10. shows that the 10-week group's collagen staining intensity in the portal areas is comparable to that of the 4-week group. Stain: Masson trichrome, x40.

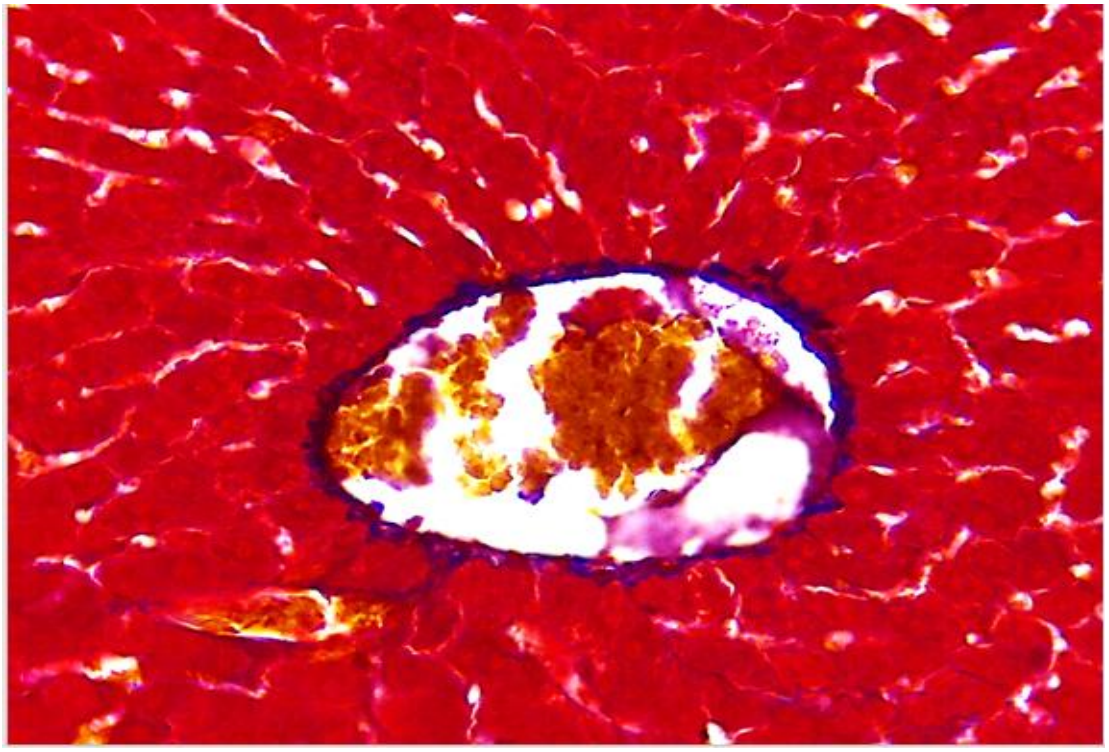


Figure 4.6.11. Group of ten weeks: Around the v centralis, discolored collagen strands are typically visible. Stain: Masson trichrome, x40.

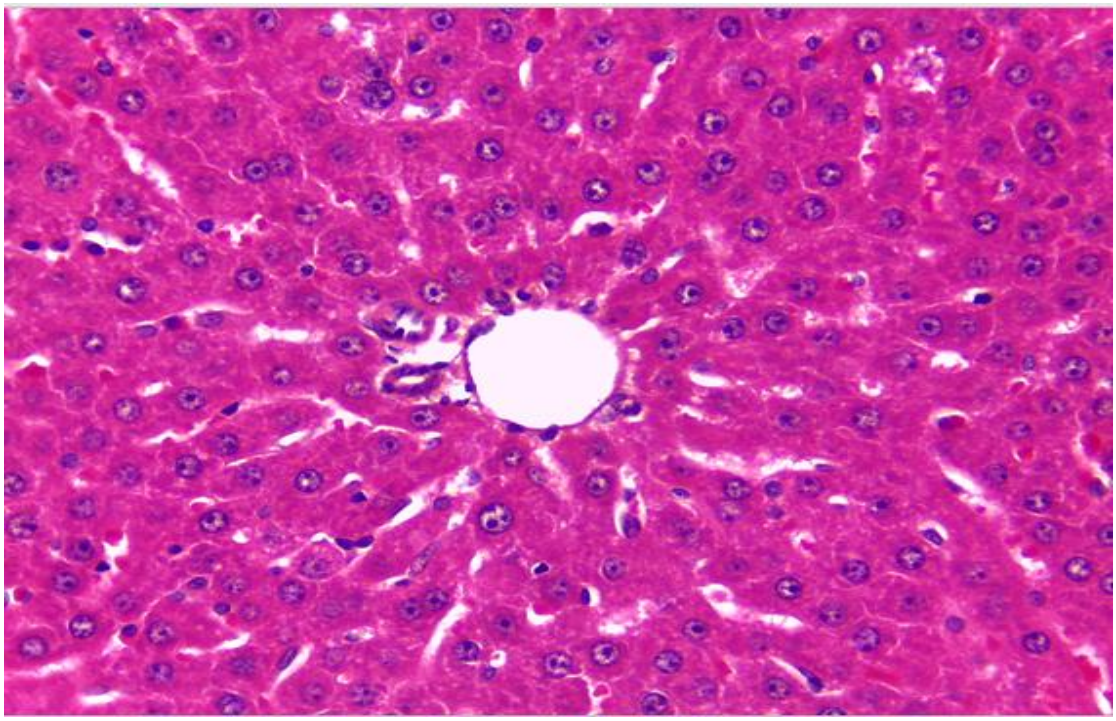


Figure 4.6.12. Group of 18-month-olds: It's evident that the hepatocytes surrounding the portal area have a typical structure. Hematoxylin and eosin staining, 40 ×.

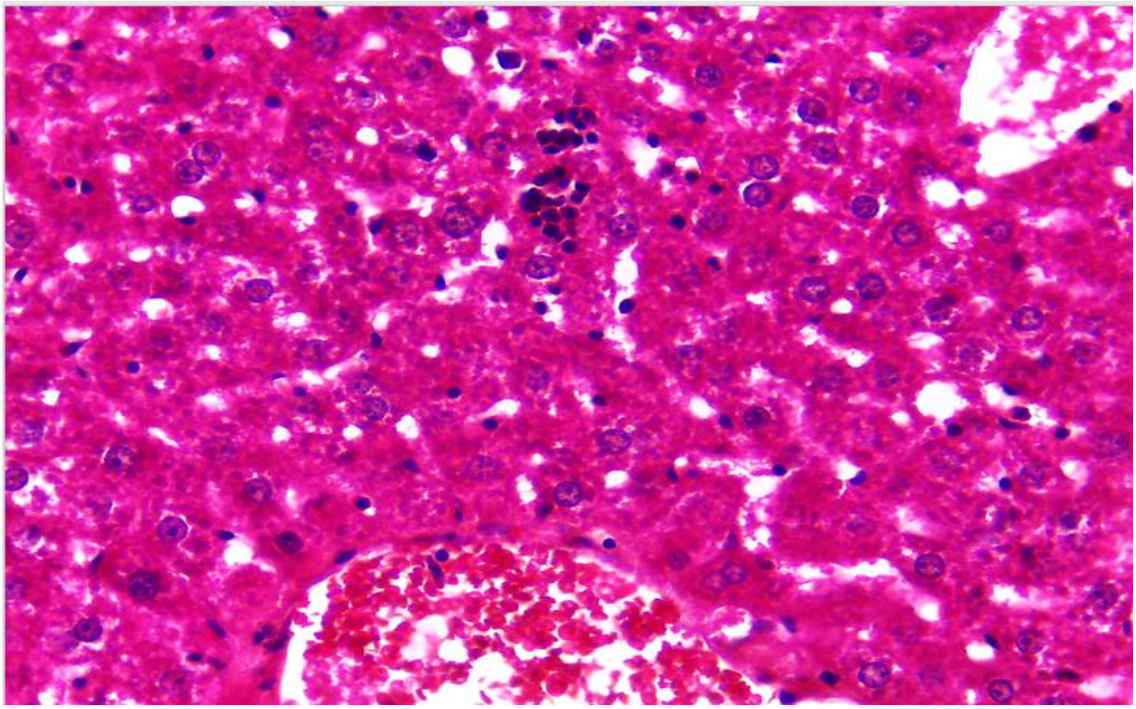


Figure 4.6.13. The central vein in the 18-month-old group has a higher concentration of mononuclear cells. x40 Hematoxylin and Eosin staining.

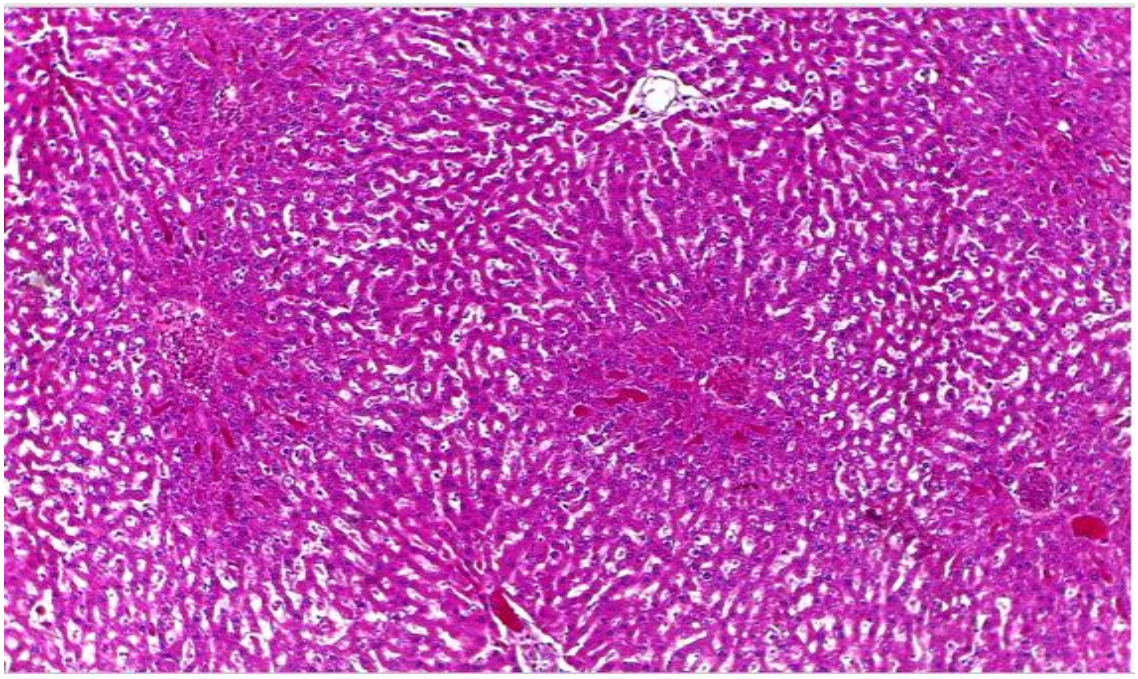


Figure 4.6.14. The 18-month-old group shows that some participants have congestion, a noticeable expansion of the sinusoids, and degeneration of the liver structure. x10 hematoxylin and eosin staining.

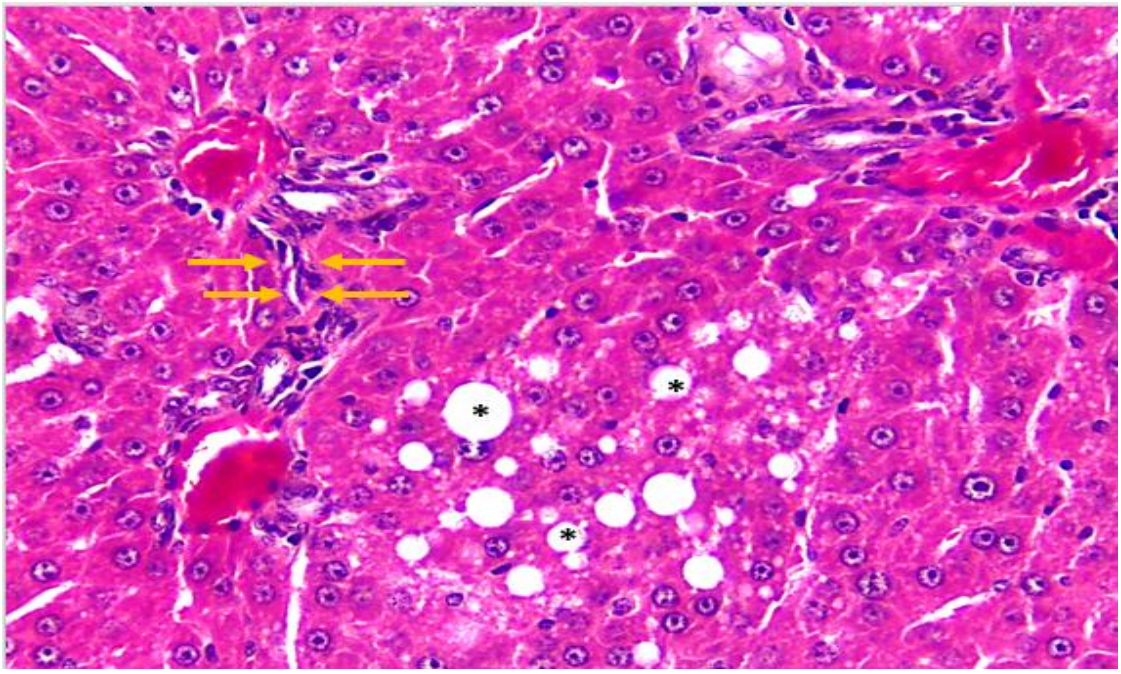


Figure 4.6.15. The 18-month-old group exhibits considerable vacuolization (*) (steatosis), bile duct proliferation in portal locations, bridging between areas (yellow arrows), and binucleated hepatocytes in certain areas. x40 Hematoxylin and Eosin staining.

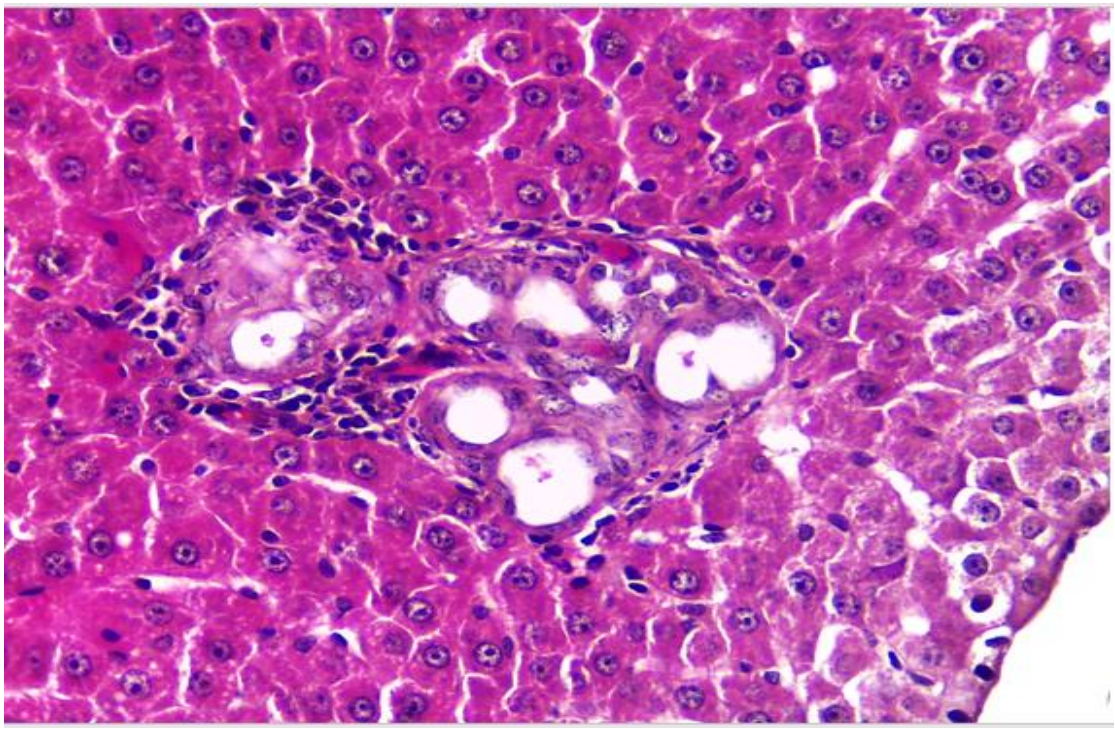


Figure 4.6.16. Group of 18-month-olds: The portal region exhibiting bile duct growth and cell expansion is observed. x40 Hematoxylin and Eosin staining.

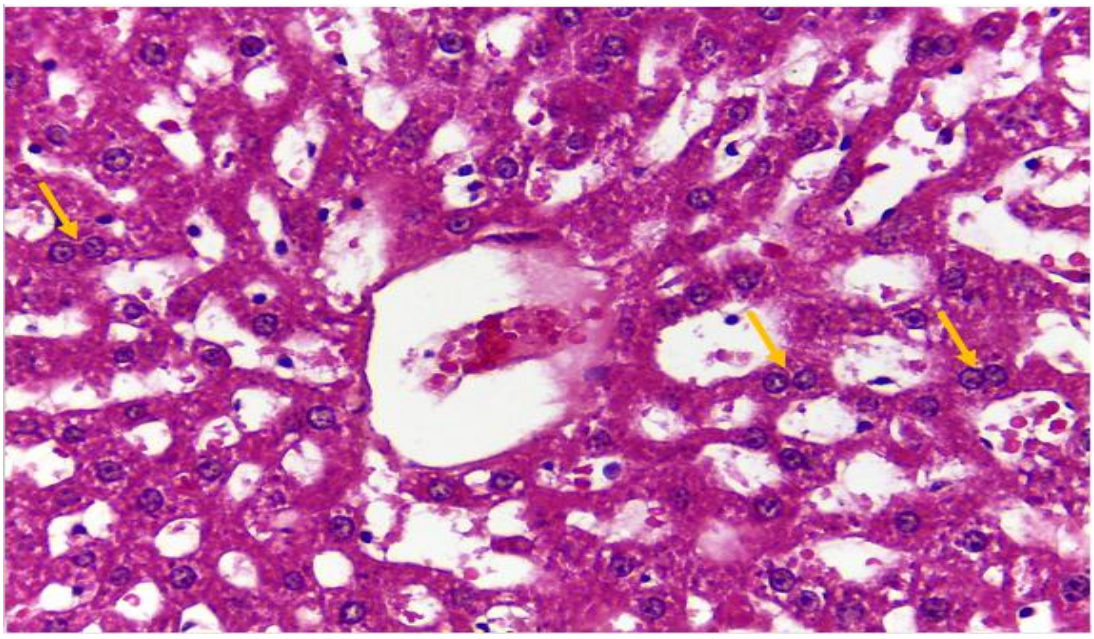


Figure 4.6.17: Around the vena centralis, there is an expansion of the sinusoids and binucleated hepatocytes (yellow arrows) in the 18-month-old group. x40 Hematoxylin and Eosin staining.

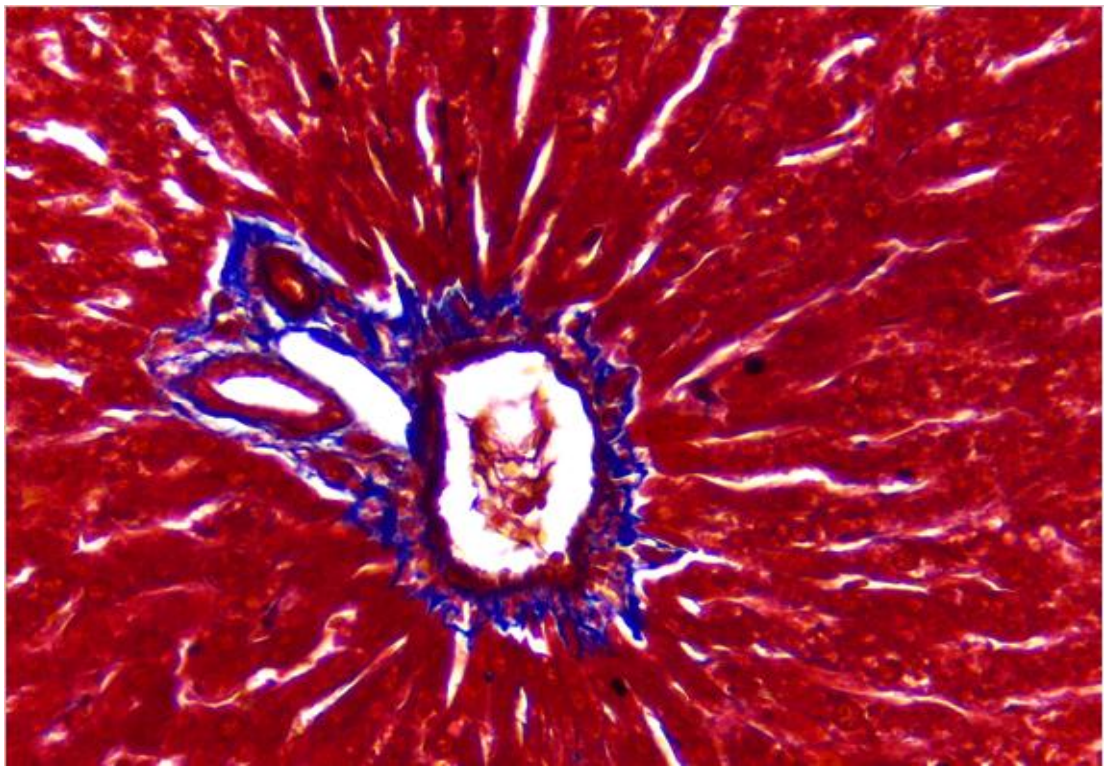


Figure 4.6.18. The 18-month-old group shows an increase in collagen fibers in the portal region. Stain: Masson trichrome, x40.

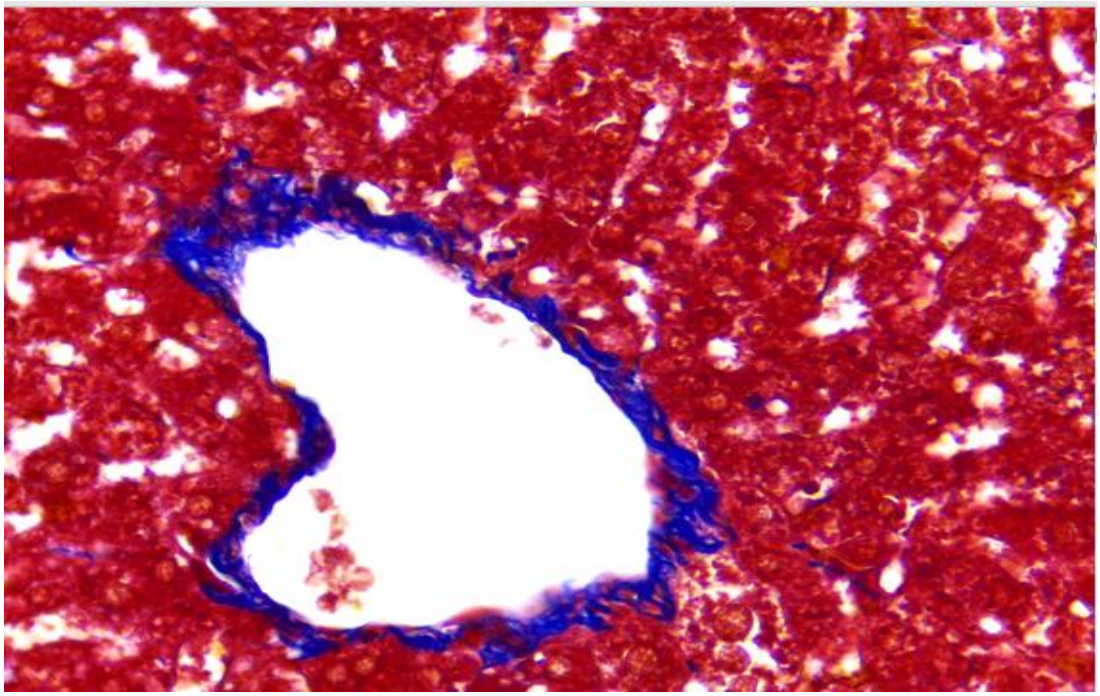


Figure 4.6. 19. Comparing the 18-month-old group to the 4- and 10-week-old groups, it is evident that the collagen fibers around the vena centralis have grown. Stain: Masson trichrome, x40.

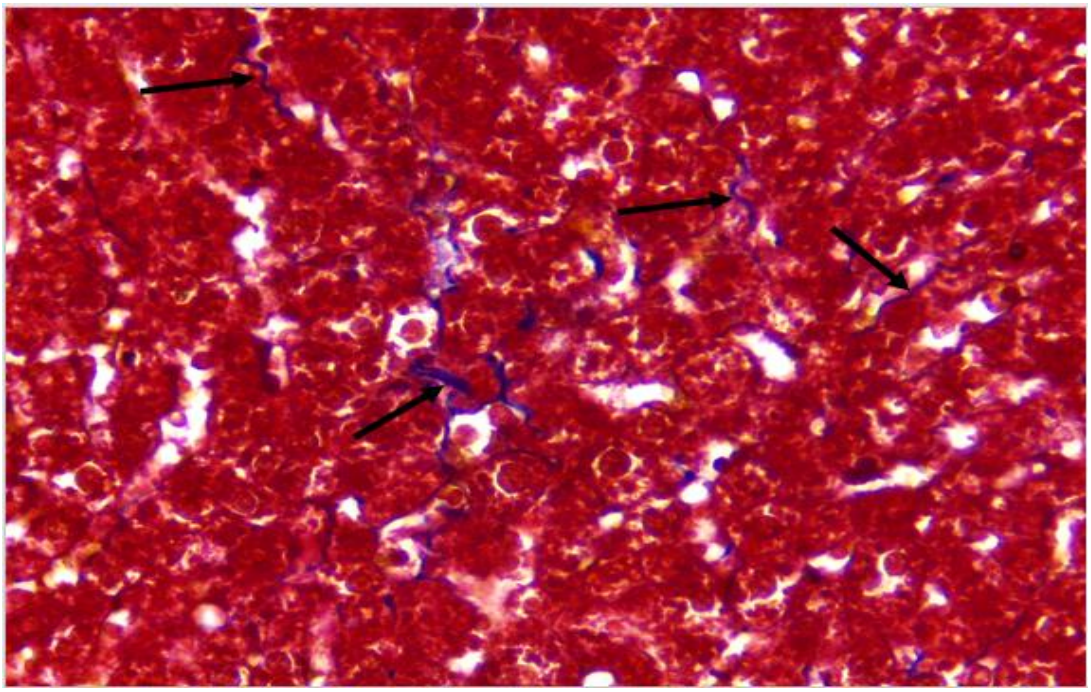


Figure 4.6.20. The 18-month-old group shows a noticeable collagen fiber staining around the sinusoids and in between the hepatocytes (black arrows). Stain: Masson trichrome, x40.

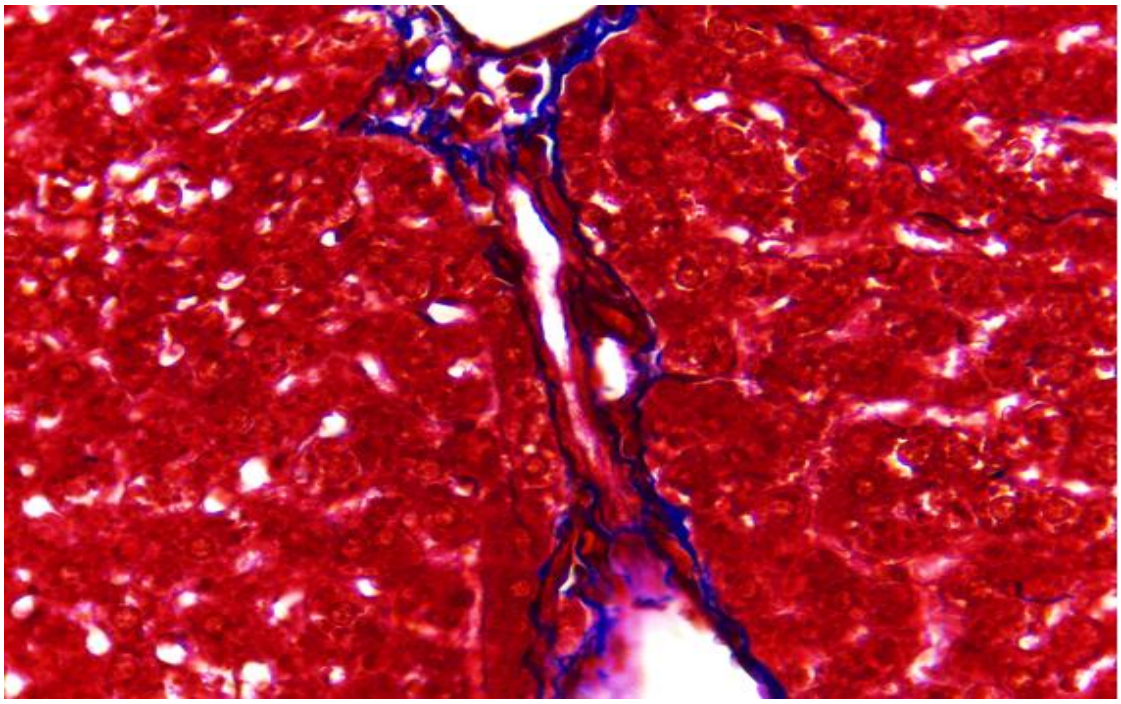


Figure 4.6.21. Between the portal areas are bridging collagen fibers, a sign of fibrotic alterations in the 18-month-old group. Stain: Masson trichrome, x40.

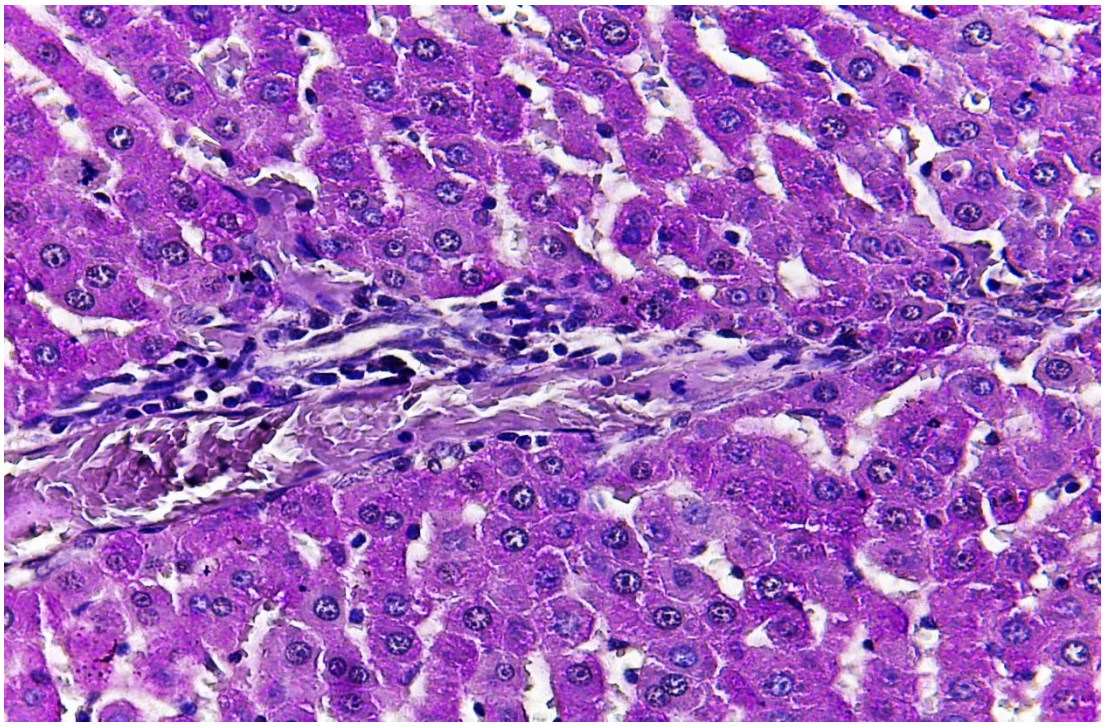


Figure 4.6.22. In 4-week-old rats, the distribution of glycogen in the hepatocytes around the portal area appears to be normal. PAS stain, 40x

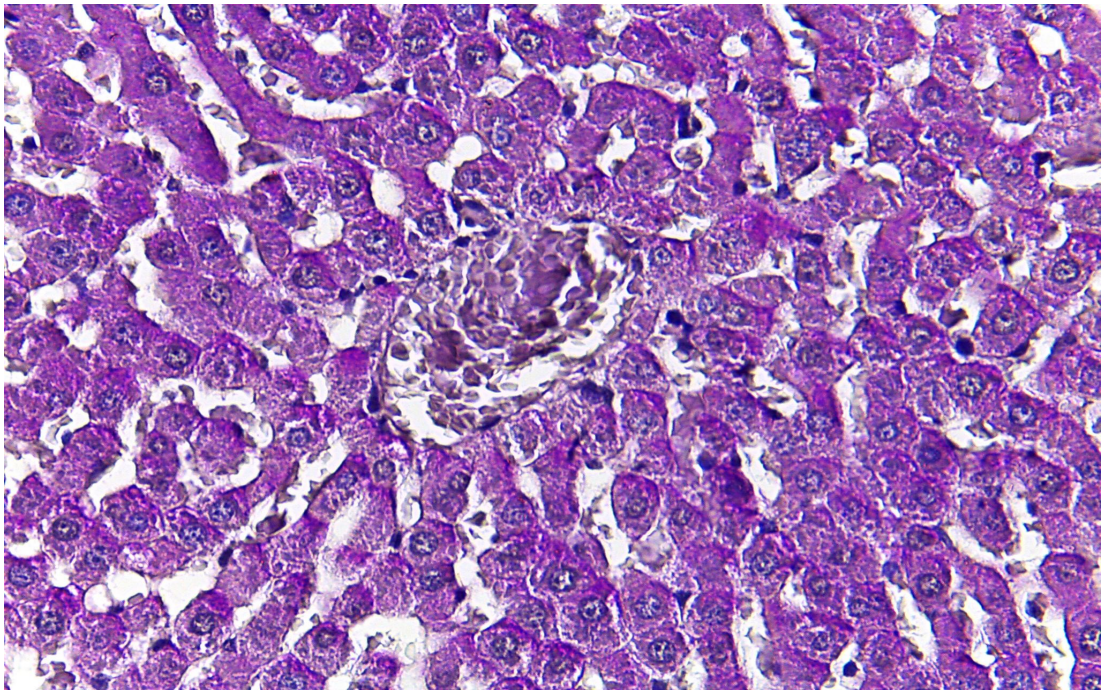


Figure 4.6.23. in the liver of a 4-week-old rat. Hepatocytes around the v.centralis have a comparable distribution of glycogen to those surrounding the portal region. PAS stain, 40x.

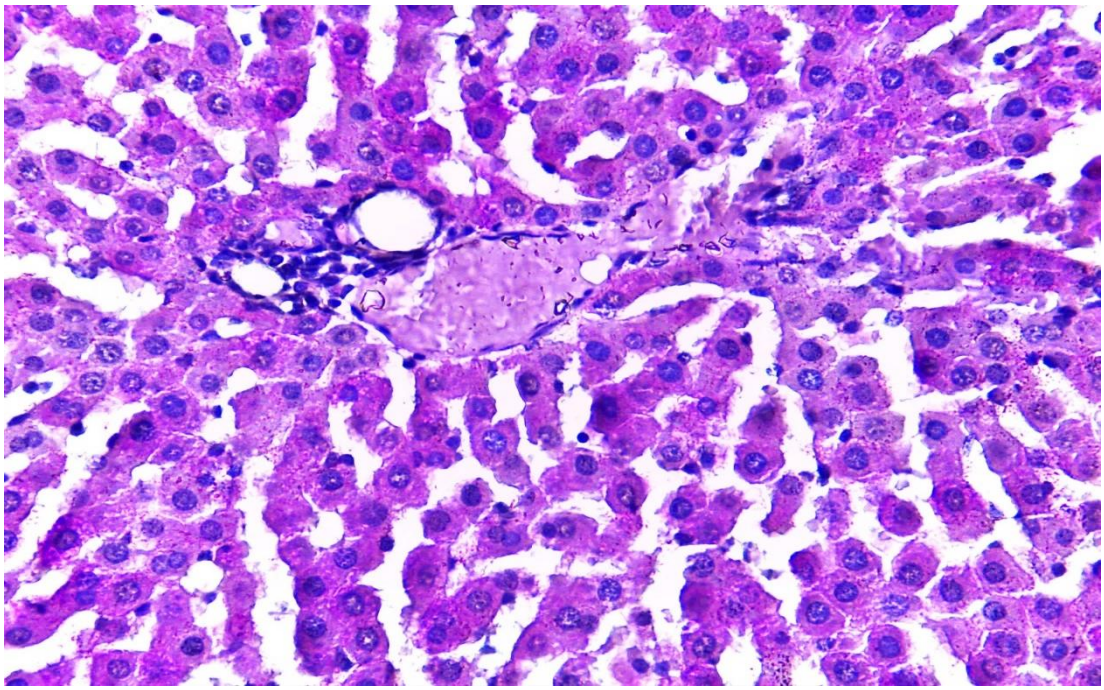


Figure 4.6.24. Rats that are 10 weeks old have their hepatocytes' glycogen distribution around the portal region visible. PAS stain, 40x.

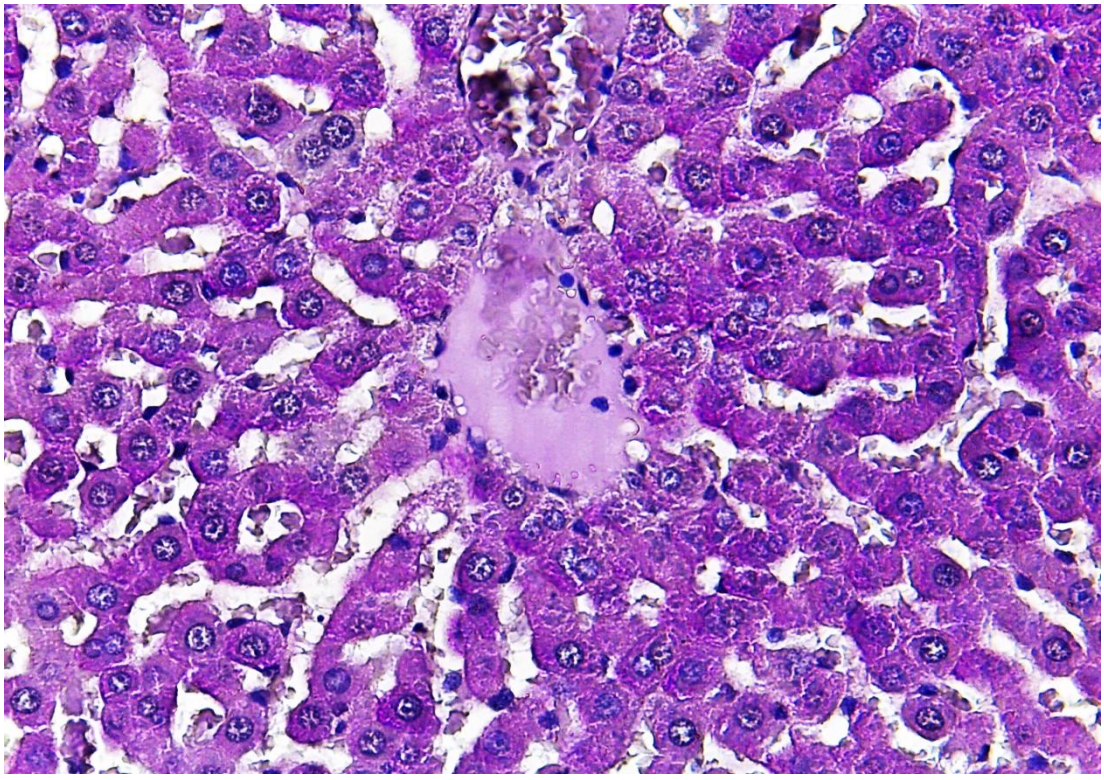


Figure 4.6.25. in the liver of 10-week-old rats. Hepatocytes surrounding the v.centralis have a distribution of glycogen. PAS stain, 40x.

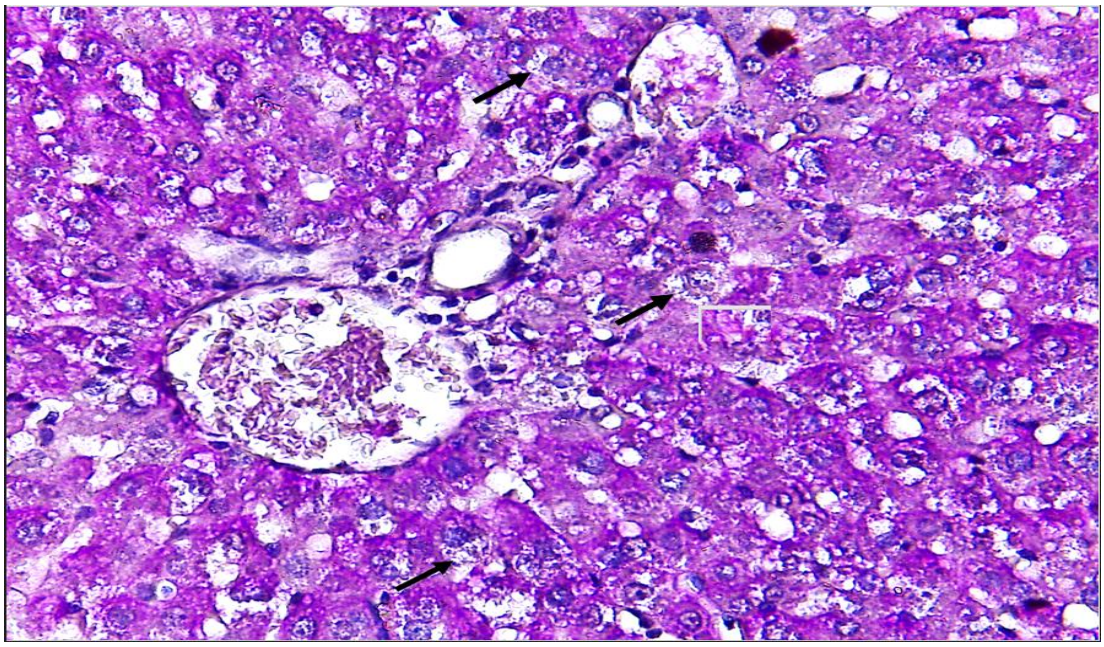


Figure 4.6.26. Compared to the 4- and 10-week-old groups, the distribution of glycogen in the hepatocytes surrounding the portal area in the 18-month-old rat liver is observed to be different, and in some of them, glycogen is either greatly reduced can be seen(arrows). PAS stain, 40x.

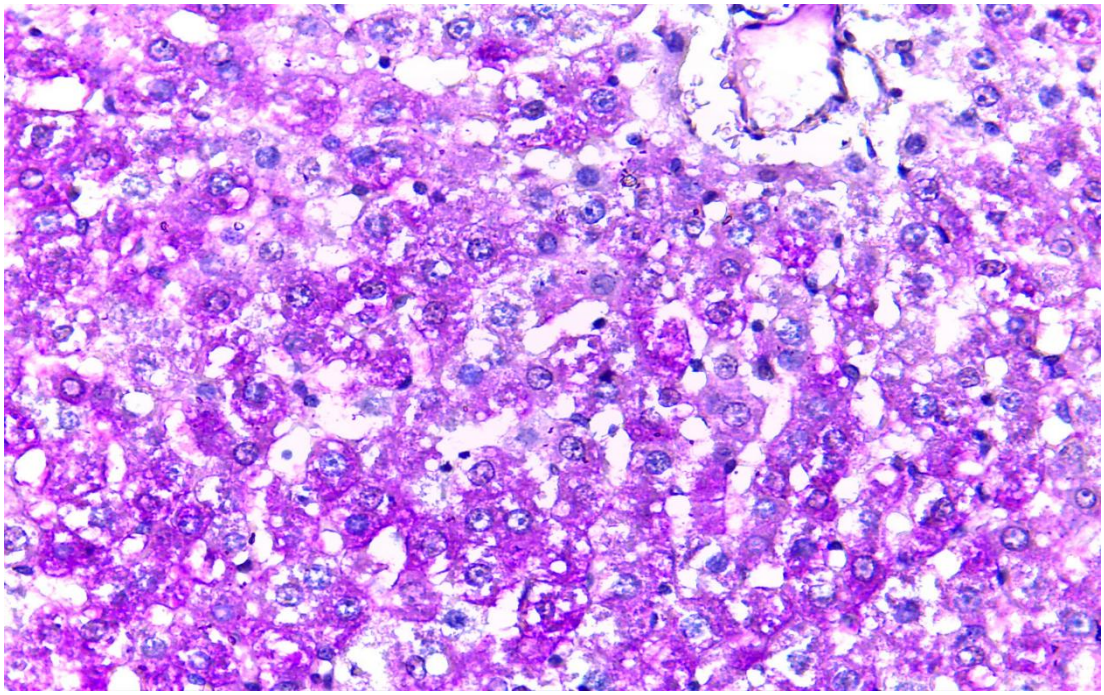


Figure 4.6.27. It has been noted that the distribution of glycogen in the hepatocytes around the portal region and central vein in 18-month-old rats is comparable. PAS stain, 40x.

Discussions

Histology studies, mainly at the tissue or cellular level of microscopy, provide excellent views regarding cellular and structural changes in the liver. Biochemical evaluations may show changes in metabolic and enzymatic activities that could accompany aging (Wang et al., 2023). There has been strong interest in the molecular mechanisms underlying these changes, with specific reference to apoptosis, autophagy, and inflammation, all of which have been implicated (Zhao et al., 2022).

Apoptosis is defined as a programmed and tightly controlled death of cells to maintain cellular homeostasis. The expression levels of caspase-3, commonly referred to as the key effector in the apoptosis pathway, are highly indicative of the level of apoptosis taking place among the liver tissues (Wang et al., 2023). Another significant process is autophagy, which is also under the control of the mammalian target of rapamycin. It is similarly another essential process that helps eliminate damaged organelles and proteins, thus playing a protective role against aging. In contrast, inflammation is often associated with the aging and chronic disease process (Zhao et al., 2022). Proinflammatory cytokines include tumor necrosis factor-alpha interleukin-6 (IL-6), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappa B), among others, that are usually upregulated in aged tissue and further contribute to hepatic dysfunction (Stahl et al., 2018).

In Table 4.1 there is significance in the variation of body and liver weights among rats at different ages as illustrated by the p-values (<0.0001). The change in body weight is significant among the age classes; that for the 18 months group is more than twice that for the 4-week group and significantly higher than the 10-week group. likewise, 10-weeks group is higher than 4-weeks age. Interestingly, despite this significant increase in body weight, liver weights do not show a corresponding trend. The weight of the liver is relatively stable across age groups: all this suggesting that the growth of the liver doesn't pace with that of the body in aging rats. This very disparity between body growth and liver size might already point to changes in body composition and organ development over time that are not linearly related.

According to table 4.2.1. the biochemical analysis across different rat groups of age 4 weeks, 10 weeks, and 18 months shows a considerable change in the lipid profile

and Liver enzyme level, which is the index of metabolic and physiological changes occurring with aging. The levels of cholesterol increased significantly from 59.17 ± 7.731 mg/dl in the 4-week-old rats to 70.17 ± 7.653 mg/dl in the 10-week-old rats before finally running up to 113.3 ± 15.36 mg/dl at 18 months with a statistically significant p-value of <0.0001 across these age groups. Triglycerides increased from 77.83 ± 13.15 mg/dl in the 4 weeks group, decreasing slightly to 68.17 ± 45.80 mg/dl in the 10-week-old rats before significantly increasing to 121 ± 45 mg/dl in the oldest group, with a p-value of <0.0001 indicating extreme significance for age-related increase. In addition to that, the liver enzymes, markers for liver health, showed a progressive elevation with age. Levels of AST increased from 95.67 ± 11.41 U/L in the 4-week-old group to 111.8 ± 21.92 U/L in the 10-week-old group and reached up to 136.5 ± 28.89 U/L in the 18-month-old group with a p-value less than 0.0001. ALT levels were 51.83 ± 5.565 U/L, remained relatively stable in the 10-week group at 51.17 ± 23.30 U/L, but increased significantly to 85.33 ± 16.16 U/L in the 18-month group with a p-value<0.0001. ALP levels significantly rise from 204.7 ± 32.20 U/L in the 4 weeks rats to 283.5 ± 42.93 U/L in rats aged 10 weeks and continue further up to 410.2 ± 90.15 U/L in old rats at 18 months with a p-value of <0.0001. These results not only suggest a significant metabolic shift but also show a likely stress in liver function with age due to increases in liver enzymes for enhanced cellular turnover or liver stress that may be associated with structural changes like fibrosis or accumulation of fat. The steep rise in ALP may indicate changes in biliary function or bone metabolism, which is closely linked to liver health.

In table 4.3.1: In rats, the changes with age in the incidence of double nucleated hepatocytes were clearly shown. Hepatocytes with two nuclei were meticulously counted in 10 different regions of liver tissue sections under 40x magnifications. At 4 weeks old, rats showed a lower average of double nucleated hepatocytes of 1.983±1.112, which significantly increased in the 10-week group to 4.967±2.178 and continued with an increase in the 18-month group to 8.433±3.734. This shows that this progressive increase, confirmed by a very significant p-value (<0.0001), would suggest that, with age, there appears to occur the rise in cellular anomalies or adaptations probably due to cellular stress, aging processes, or regenerative response taking place within the liver of rats.

In table 4.4.1. Histological examination of rat liver tissue sections at different ages 4 weeks, 10 weeks, and 18 months reveals significance changes in liver structure and function with age, illustrated by scoring on the sections at 40× magnification. It was scored that from the 4 weeks group to the 18 months group, there is progressive sinusoidal dilation and congestion. Indicating potential complications such as compromised blood flow or increased hepatic pressure in 18 months group. Also, the number of mononuclear cells increases with age, which might indicate increased inflammation or immune response within the liver, probably a sign of ongoing hepatic injury or repair. More significantly, vacuolization and lipid accumulation increase from none in the 4 weeks rats to very high values in the 18 months rats, indicating metabolic stress or probably the first process of fatty liver disease. It is also evident that fibrosis progresses with age, where there is substantial development of the fibrotic tissue in the 18 months which is the oldest rat. This is probably because of chronic inflammation or repetitive liver cell injury. finally, the proliferation of bile ducts is progressively increasing with age and may represent some reactive or compensatory response to obstruction of bile flow or damage to the liver cells.

In Table 4.5.1.1 with figure 4.5.1.1.1. It has been shown that Interleukin-6 (IL-6) levels increase with age. This is so because of the mean levels recorded in the different stages of life: 1.354 in 4-week-old rats, 3.912 in 10-week-old rats, and 5.155 in 18-month-old rats. Statistical analyses further support these findings: there were significant increases in the IL-6 levels between the youngest and both older groups according to p-values of 0.0230 and 0.0075, respectively; no significant difference was seen between the 10-week and 18-month groups with a p-value of 0.1414. This pattern may set the stage for a more pronounced increase in IL-6 levels earlier in life. It could have implications for the role of this cytokine during early immune system development or in the body's inflammatory response as it matures.

In Table 4.5.2.1 and figure 4.5.2.1.1. show a significant increase in M-TOR levels with age in the rat groups. The youngest group, 4 weeks, was 1.346. Then, at 10 weeks, it jumped drastically to 3.830 in the 10-week group and an even more dramatic increase to 6.823 in the 18-month-old group. Statistically, the increase in M-TOR levels from the 10-week to the 18-month group was significant at p=0.0366,

and from the 4-week to the 18-month group, it was at p=0.0067; thus, it progressively and significantly increased with age. However, there was no statistical significance between the 4-week and 10-week groups, as this variance only reached p=0.0595. This pattern demonstrates the potential role of M-TOR in regulating developmental autophagy and aging processes.

There is an age-dependent increase in Caspase 3 across the different age groups of rats according to table 4.5.3.1 and figure 4.5.3.1.1. The mean level is 1.054 for the 4-week-old rat group and significantly goes up to 5.523 in the 10-week-old group and further to 8.414 in those at 18 months, with highly significant p-values less than 0.0003. These results further underscore the role of Caspase 3 in terms of the aging process and almost certainly reflect an increase in apoptotic activity as the organisms mature.

In table 4.5.4.1 and figure 4.5.4.1.1. The NF-kappa gene expression data shows a significant increase with age, demonstrating statistically significant changes between the 4-week-old and 10-week-old groups and also between the 4-week-old and 18-month-old groups. More specifically, NFKB levels increased from an average of 1.187 at four weeks to 8.318 at ten weeks and rose slightly, to an average of 9.019 at 18 months. The fact that there is a substantial increase from the 4 weeks group to the 10 weeks group, and which then stabilizes into 18 months, would suggest that NFκB has some critical role in early developmental processes and perhaps the maintenance of certain physiological functions into adulthood. This expression profile could be taken to indicate that NF-κB is acting to control immune responses or inflammation as part of aging.

In Light Microscopic findings, Examining the liver tissues of 4-week-old prepubertal rats, the classical liver lobule structure, portal regions, etc. Figures 4.6.1 and 4.6.2 show the normal structure of the centralis, sinusoids, hepatocyte cell cords, and Kupffer cells. Increased mononuclear cell counts were seen in certain regions, however they were uncommon (Figure 4.6.3). In each field, binuclear hepatocytes were found. Examining Masson trichrome staining’s, it was possible to see no collagen growth surrounding the centralis in the portal area and v (Figure 4.6.4, Figure4.6.5).

Hepatocytes and portal regions in the adult 10-week-old rat liver tissue were largely comparable to those in the 4-week-old group. In this group, there was an increase in the number of binucleated hepatocytes (Figure 4.6.6, Figure 4.6.7). Unusual v. Figure 4.7.8 showed vacuolization indicative of steatosis in the hepatocytes and congestion in the sinusoids surrounding the centralis. Bile duct proliferation was observed in the portal area in certain patients (Figure 4.6.9). Staining with Masson trichrome in entry regions and v. Collagen levels surrounding the centralis were comparable to those of the 4-week group (Figure 4.6.10, Figure 4.6.11).

Certain liver tissue samples from 18-month-old rats showed normal liver architecture (Figure 4.6.12), but higher populations of mononuclear cells were observed throughout (Figure 4.6.13). Certain participants exhibited disruptions to the typical lobule architecture, including significant enlargements of the sinusoids, congestion, and bile ducts in portal locations (Figure 4.6.14). Proliferation, extensive vacuolization, and bridging across partial sections were noted (Figure 4.6.15). Proliferation of the bile ducts was frequent (Figure 4.6.16). Greater numbers of binuclear hepatocytes resembled those of the 10-week group (Figure 4.6.17). Collagen was found in greater amounts surrounding the portal area and the central vein (Figure 4.6.18); between the hepatocytes and around the sinusoids (Figures 4.6.19 and 4.7.20); and between the portal areas, where bridges were formed, as shown by Masson staining (Figure 4.6.21).

The older population exhibited ischemia findings, necrotic areas, multiple portal sites with bile duct proliferation, extensive fat-induced vacuolization, and fibrotic alterations as a result of increased collagen fibers. The 4- and 10-week groups also showed elevated mononuclear cells, despite their rarity. In the 10-week group, relatively few locations showed signs of bile duct proliferation and vacuolization.

In 4-week-old (Figures 4.6.22, 4.6.23) and 10-week-old (Figures 4.6.24, 4.6.25) rat hepatocytes, the glycogen distribution was found to be homogenous, and the staining in the hepatocytes surrounding the portal area and central vein was identical. The hepatocytes of 18-month-old rats, which we regarded as the elderly group, showed decreased glycogen distribution in the portal area and surrounding the central vein, and several hepatocytes showed no staining (Figures 4.6.26, 4.6.27).

CHAPTER V

Conclusion and Recommendation

Conclusion

A study on aging liver histology in rats undertook minute observations of the differences in histological and molecular parameters in liver tissue samples from various age-specific rats: prepubertal, young, and old. In the view of this research, age significantly changes concerning effects on the liver and how aging has an exciting influence on the expression pattern of critical biochemical and molecular markers. Notable findings from this study included apparent structural changes within liver tissues of aging rats; that is, variations in liver architecture or cellular composition were predominantly affecting hepatic function. Biochemical markers of apoptosis showed extremely high values for the older rats, significantly increasing with joints, thus showing an increase in apoptotic activity during senescence. Given that high expression of mTOR is linked with enhanced autophagy and using observation from the study that aging livers demonstrated mounts in mTOR expression, it turns out that altered autophagic processes likely take part. Consequently, this means higher expression of pro-inflammatory cytokines like IL-6, and NF-κB in older rats, which argues for an increased inflammatory response with age that builds a basis for impaired liver function rather frequently. More specifically, by fold change analysis, it has been shown that minimal expression in those markers at four weeks was enormously increased at ten weeks and 18 months. This database reveals progressive enhancement of apoptotic, autophagic, and inflammatory processes with age. These molecular changes have essential implications for liver function and can lead to age-associated liver disease and liver weakening. That is because it has been pointed out that the process of liver aging is so complex it leads to structural and functional alterations that are detrimental to liver health. Thus, there

is a need to investigate in much more detail the molecular mechanisms underlying liver aging.

Recommendation.

There are some recommendations for further research in addition to other measures into the impacts of aging on liver health:

To begin with, the maintenance of in-depth studies longitudinally to trace how histological and molecular changes went on in liver tissues over time. This would be informative about dynamics in the process of liver aging and indicative of crucial intervention times.

In addition to that, identify any influence from the outside caused by diet, environmental stimuli, and toxicants on liver aging. The knowledge of such interactions could be used to minimize external risks and to promote health/hepatoprotection.

Furthermore, that means exploring possible interventions—dietary changes, pharmacologic treatments, lifestyle modifications—including those that may help lessen the harmful impacts of aging on the liver. The strategies include testing antioxidants, anti-inflammatory drugs, and caloric restriction.

finally, stretch this research onto different animal models and further, in case it is possible, onto human liver tissues to sort out the generalizability of the findings. This will help translate animal studies into applications for human health by making comparative studies.

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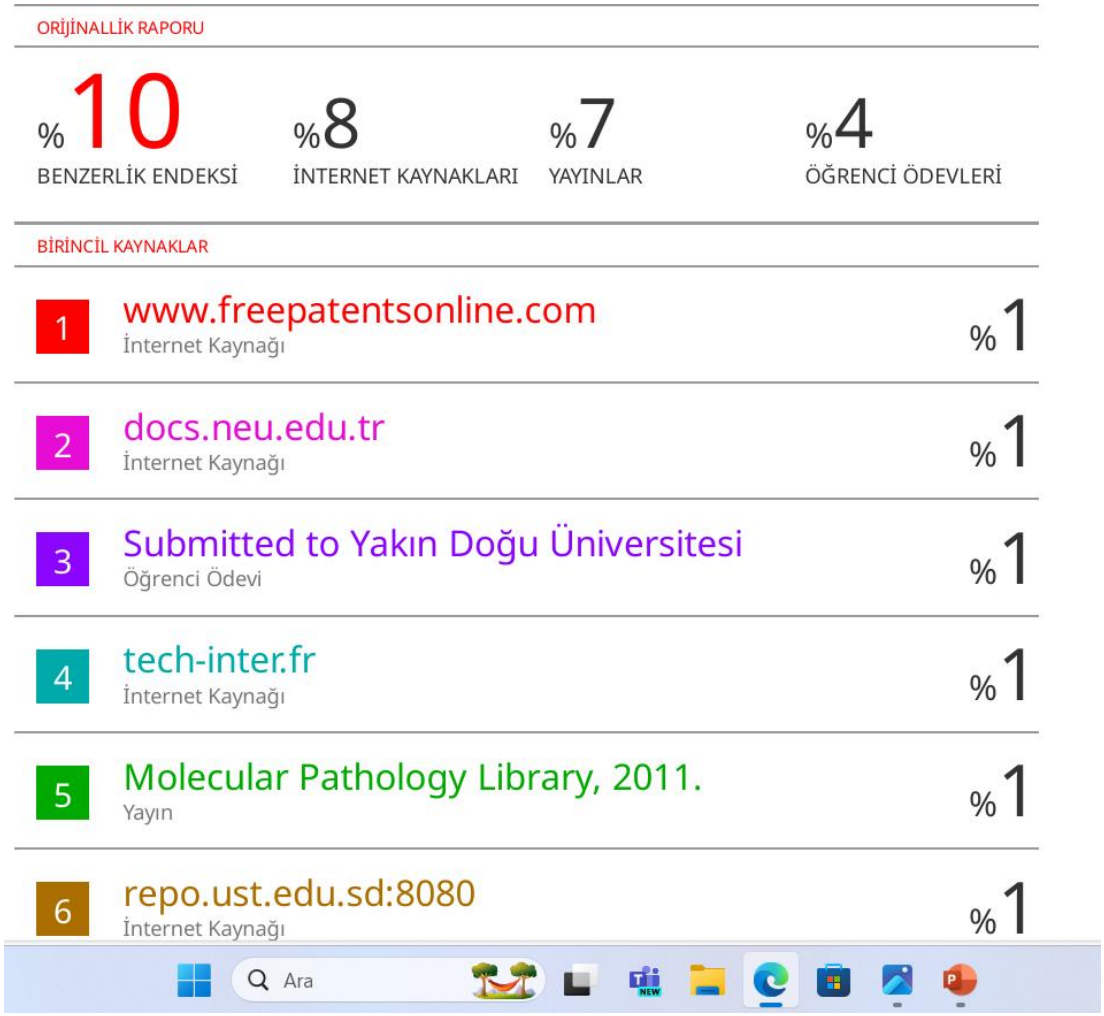
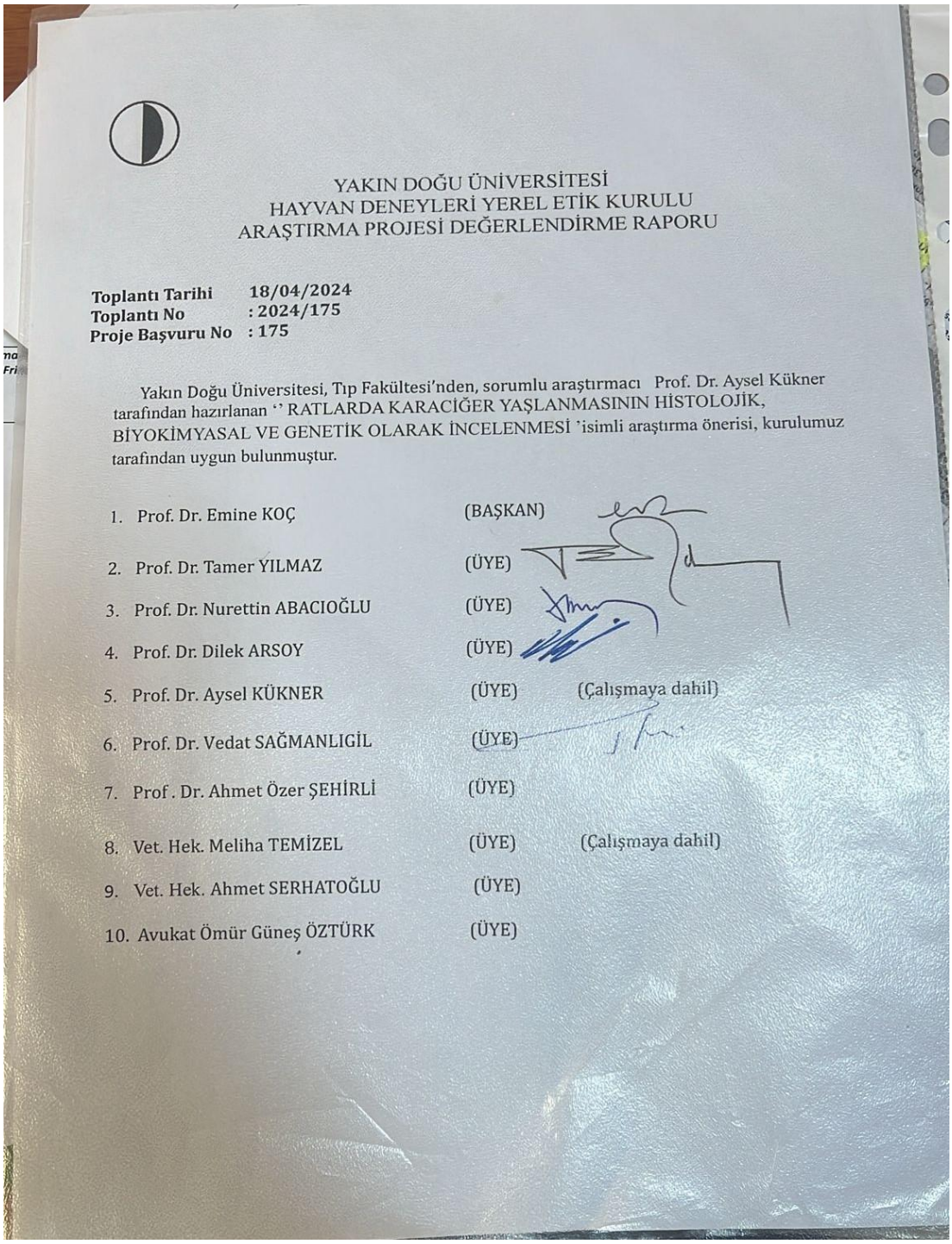
APPENDICES

similarity report



Ethic report



YAKIN DOĞU ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi 18/04/2024
Toplantı No : 2024/175
Proje Başvuru No : 175

Yakın Doğu Üniversitesi, Tıp Fakültesi'nden, sorumlu araştırmacı Prof. Dr. Aysel Kükner tarafından hazırlanan '' RATLARDA KARACİĞER YAŞLANMASININ HİSTOLOJİK, BİYOKİMYASAL VE GENETİK OLARAK İNCELENMESİ 'isimli araştırma önerisi, kurulumuz tarafından uygun bulunmuştur.

1. Prof. Dr. Emine KOÇ (BAŞKAN)
2. Prof. Dr. Tamer YILMAZ (ÜYE)
3. Prof. Dr. Nurettin ABACIOĞLU (ÜYE)
4. Prof. Dr. Dilek ARSOY (ÜYE)
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