v. 2, n. 41, 2022

International Journal of Health Science

COMPARATIVE STUDY OF THE LIVER STRUCTURE BETWEEN YOUNG, ADULT AND ELDERLY RATS

Andréia Affonso Barretto Montandon
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara – São Paulo, Departamento de Odontologia Social

Eleny Zanella Balducci
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara – São Paulo, Departamento de Morfologia

José Paulo de Pizzol Júnior
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara – São Paulo, Departamento de Morfologia

Cleverton Roberto Andrade
Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara – São Paulo, Departamento de Fisiologia e Patologia

Abstract: The aim of this study was to analyze the morphological aspects of the liver parenchyma components in three stages of age in Holtzman rats. The livers of 33 female rats (*Rattus norvegicus* albinus, Holtzman) aged 120 days (G1 - young), 180 days (G2 - adults) and 540 days (G3 - elderly), with natural aging were studied. The material was processed and submitted to picrosirius technique and polarized light for visualization of collagen fibers, Periodic Acid - Schiff Reactive (PAS) to evidence the glycogen and stained nuclei to search for glycogen and mucus substances. After obtaining the percentage of birefringent collagen, a two-way analysis of variance (TwoWay ANOVA) was performed followed by Tukey's post-test ($p<0.05$) and for collagen, the analysis of variance was the One-way ANOVA. Morphological analysis of the liver showed normal features of the lobular parenchyma of the liver (hepatocytes, sinusoid capillaries, collagen fibers and blood vessels), but with mild inflammatory infiltrate in the portal space in some animals. Morphometric analysis did not reveal differences between groups regarding the frequency of nuclei and sinusoid capillaries, although this occurred in the "others" parameter between G1 and G2. Glycogen was present in all three groups. G1 presented the smallest amounts of birefringent collagen fibers, followed by G2; G3 showed significantly higher values of birefringent collagen fibers. The results obtained allowed us to conclude that the most relevant alterations obtained during the process of senescence of the rats' liver referred to the more accentuated presence of collagen fibers.
Keywords: Aging, senescence, mice, liver, collagen.

INTRODUCTION

The term aging is used to designate the morphofunctional changes that progressively compromise the ability of individuals to respond to the environment and to maintain homeostasis (JECKEL-NETO E CUNHA, 2011).

The liver appears to age reasonably well compared to other organs, as its homeostatic functions are not seriously compromised and liver function remains in senescent individuals (TIETZ ET AL., 1992). It is one of Organs most studied organs in aging due to its metabolic aspects, structural changes (FERRIOLI et al., 2011), and especially its high regenerative capacity (FAUSTO et al., 2006; MICHALOPOULOS, 2007). However, this capacity for liver regeneration decreases with aging, due to a decrease in the cell cycle and an increase in autophagy and apoptosis of liver cells (SERRA et al., 2015; FERNÁNDEZ-GARCIA et al., 2018), in addition to a deregulation of liver cells. biological systems that lead to an increase in oxidative stress and inflammation, according to FERNÁNDEZ-GARCIA et al. (2018).

As the largest gland in the human body, the liver is covered by a thin connective tissue capsule, thicker in the hilum region, where the portal vein and hepatic artery penetrate. The right and left hepatic ducts and the lymphatics leave the liver also in the hilum region (JUNQUEIRA, CARNEIRO, 2013).

The classic way to describe the functional unit of the liver involves the concept of the so-called hepatic lobule with epithelial cells arranged in plates, called hepatic cells or hepatocytes, which are radially arranged in the hepatic lobe, arranged like small bricks of a wall, supported by a delicate network of reticular fibers. These cell plates are directed from the periphery of the lobe to its center and anastomoses freely, forming a labyrinth. The spaces between these plates contain capillaries, the hepatic sinusoids, irregularly dilated vessels, which transport the blood that arrives through the branches of the portal vein and the hepatic artery and the so-

called spaces of Disse; the bile canaliculi that transport bile to the bile ducts in the portal spaces (GERBER, THUNG, 1987).

The sinusoids contain macrophages, called Kupffer cells, which will perform several functions (HUANG, LIAW, 1995). Kupffer cells represent the so-called Mononuclear Phagocytic System in the liver and proliferate in several nonspecific situations, when there is hepatic or systemic aggression (GERBER, THUNG, 1987), and their hyperplasia is described in thyrotoxicosis (HUANG, LIAW, 1995; BOGLIOLO), 1994). In the rat liver, 96% of its structure is formed by the lobular parenchyma and only 4% is formed by the non-lobular parenchyma (ENGELMAN et al., 2001; WEIBEL et al., 1969).

The liver plays a central role in carbohydrate metabolism, and glycogen is an important component of the liver cell, which at electron microscopy appears in the form of coarse granules, generally located in the smooth endoplasmic reticulum zone, functioning as a deposit that hepatocyte mobilizes when hypoglycemia occurs (MULLER, SEITZ, 1984a; MULLER, SEITZ, 1984b). Glycogenic liver disease was a term proposed by Torbeson (TORBENSON, CHEN et al. 2006) and represents the accumulation of glycogen in hepatocytes, being a poorly recognized complication of long-standing poorly controlled diabetes mellitus, characterized by abnormal accumulation of glycogen in hepatocytes, elevation of liver enzymes and hepatomegaly (TORBENSON et al., 2006). Staining with Periodic Acid Schiff (SUPUTTAMONGKOL et al., 2003) allows the identification of these accumulations (HUDACKO, MANOUKIAN et al. 2008). Thus, among the functions performed by the liver are the exocrine and endocrine glandular function, immune function, formation and secretion of bile (XAVIER, 2011).

Liver aging includes macroscopic, histological, and physiological changes without showing evidence of liver functions (XAVIER, 2011).

Glycogen represents the storage form of sugars in the animal cell, being stored naturally in the hepatocyte (SOARES FILHO et al., 2011). In the PAS method, the oxidation of vic-glycol groups in aldehydes by periodic acid occurs, which form complexes with the Schiff reagent, staining in red/ magenta, which makes it possible to prove the presence of glycogen in animal liver sections. (MCMANUS, 1946).

O colágeno Collagen is considered the most abundant component of the extracellular matrix of many types of soft tissues (HALPER, KJAER, 2014) and fibrosis is a characteristic of the aging of several organs, including heart and kidney (GAGLIANO et al. 2000), reflecting the increased deposition of the physiological components of the extracellular matrix. Thus, the liver's response to injury consists of fibrosis (GAGLIANO et al., 2002) and age is a critical factor that affects many immune-mediated processes, including potentially the liver injury response to injury by increasing fibrosis, which in extreme cases can lead to cases of liver cirrhosis (COLLINS et al., 2013). According to GAGLIANO et al. (2002), hepatic fibrosclerosis is mainly explained by a reduced proteolytic activity of the MMP matrix, in which TIMP-1 appears to be an important regulatory factor.

Aging is a multifactorial process that occurs differently for each individual, because biological and environmental factors are involved in this process (LIMA-COSTA, VERAS, 2003; DE SOUZA et al., 2008).

In addition, the physiological decline resulting from aging can be seen by the functional and structural imbalances of the systems, and naturally, of the organs and tissues that form these systems. Cellular

changes lead to a decrease in functional reserves, which makes the elderly more prone to diseases (DE SOUZA et al., 2008). Thus, a multidisciplinary approach is necessary for a positive interference in the effects of aging, delaying its negative effects and reducing the possibility of complications so that the elderly live in the best possible way (FREITAS et al., 2002).

According to MOTA et al. (2004) independently of other factors, the genes that control the activity of the neuroendocrine system alter their expression and condition the observed declines in hormonal regulation in aging, among them, the collapse and death of regulatory cells of the neuroendocrine system that lead to an imbalance. of body homeostasis (JECKEL-NETO, CUNHA, 2011). Such reports agree with the Neuroendocrine Theory studied by FINCH (1993), whose data support the main hypothesis that age-related changes are usually caused by specific physiological factors that are extrinsic to cells.

Young individuals with endocrine diseases present the same morphological, functional and biochemical alterations as those found in elderly individuals without diseases, confirming that there may be a neuroendocrine marker for the aging process (LIBERMAN, 2011).

Considering that aging is recognized as a progressive, gradual and variable process characterized by the increasing loss of functional reserve, it results in morphological, physiological and biochemical changes that progressively compromise the individual's ability to respond to the environment and the maintenance of homeostasis (JOHNSON et al., 2006, JECKEL-NETO, CUNHA, 2011), the study of the relationship between aging and the liver stands out due to the multifunctionality of this organ.

OBJECTIVE

To analyze the morphological aspects of the liver parenchyma components: hepatocytes, sinusoid capillaries and collagen fibers in the three age phases of Holtzman rats.

MATERIAL AND METHODS

ANIMALS, ETHICAL CONSIDERATIONS AND COLLECTION OF BIOLOGICAL SPECIMENS

The animals used were the object of a previous study called "**Histological and functional aspects of young, adult and elderly rats: comparative study",** (process CEEA FOAr 36/2010), with a further supplementary process for the present study CEEA FOAr 20/2015, allowing the use of the livers removed from the rats.

Therefore, 35 female rats were used (*Rattus norvegicus* albinus, Holtzman), from the Central Animal Facility of Universidade Estadual Paulista "Júlio de Mesquita Filho" -UNESP, Campus de Araraquara, with body weight varying between 120 g and 650 g, which were kept in individual cages and fed with granulated food and water *ad-libitum*, in an air-conditioned environment; two elderly women were not used because they had pathologies such as wounds. The animals were divided into three age groups used by LU et al, (2005 and 2008): G1- 10 young animals with 120 days of age (04 months), G2- 10 adult animals with 180 days of age (06 months) and G3- 13 aged animals with 540 days of age (18 months).

The rats belonging to G3 were obtained from the Central Animal Facility of the Campus of Araraquara at approximately seven months of age and were housed in the vivarium of the Faculty of Dentistry until aging (18 months). To establish the sample size for the experiments, the analysis of the Gauss curve was used as a criterion, which defines that within a sample the majority responds

to normality (average) and the minority to deviations (standard deviation). Thus, if the sample chosen is 10 animals, even if there were animals in this population that did not correspond to normality, we would have between 5 and 7 animals to prove the results, and this number is considered acceptable.

At the end of each period, the animals were anesthetized intramuscularly with an association of 10% Ketamine (Bayer do Brasil) and 2% Xylazine Hydrochloride (Vibac do Brasil) at doses of 0.08 ml and 0.04 ml per 100g of weight. body respectively. Then, the liver of the animals was removed and fixed in a solution containing 4% formaldehyde (prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer at pH 7.4 for 48 hours.

PROCESSING FOR LIGHT MICROSCOPY

The fixed liver was dehydrated in increasing concentrations of ethanol, cleared in xylene, infiltrated and embedded in paraffin. The 6 µm thick sections were obtained with the aid of a microtome and were stained with hematoxylin and eosin (HE) and subjected to the following histochemical reactions: picrosirius technique associated with polarized light microscope analysis according to Montes et al. (1984) for visualization of collagen fibers and Periodic Acid - Schiff Reactive (PAS), to show glycogen.

MORPHOLOGICAL ANALYZES

Morphological analyzes were performed on six animals from each group. In the sections stained by H.E, photomicrographs with 0.15mm² were taken, totaling 0.9mm² per group, to analyze the general structure of the liver, that is, hepatocytes, sinusoid capillaries and collagen fibers. Images were captured using an Olympus image capture camera (DP71) attached to an Olympus microscope (BX-51)

Using morphometric parameters, the following were quantified: 1) the number of hepatocyte nuclei, 2) the number of sinusoid capillaries, and 3) other elements. For this, a grid with 196 intersections was used.

The data obtained from the morphometry were subjected to statistical analysis using the GraphPad Prism 6.01 software (GraphPad Software, La Jolla, CA, USA). Two-way analysis of variance (TwoWay ANOVA) was performed followed by Tukey's post test for multiple comparisons, with an accepted significance level of 0.05.

HISTOCHEMICAL ANALYSIS OF GLYCOGEN

Liver sections of all animals were submitted to the periodic acid-Schiff Reactive (PAS) reaction and the nuclei were stained with Hematoxylin, to observe the presence of glycogen and mucus substances, according to the MACMANUS technique (1946).

ANALYSIS OF COLLAGEN CONTENT IN THE LIVER

To analyze the content and arrangement of collagen present in the liver, the picrosirius technique associated with polarized light microscope analysis was used, according to Montes et al. (1984). Two semi-serial slices of liver from young, adult and elderly rats were used. The sections were deparaffinized, hydrated and immersed in a concentrated picric acid solution for 5 min. After rapid washing, sections were immersed in a 0.1% Sirius-Red solution for 1 hour. Subsequently, the sections were washed, dehydrated, diaphanized and mounted for analysis under a polarization microscope.

Polarization filters coupled to an Olympus light microscope (model BX-51) were used. In each slice, 2 images were obtained with an area of 257x341µm each. With the aid of the ImageJ® (NIH) program, the bi-refringence

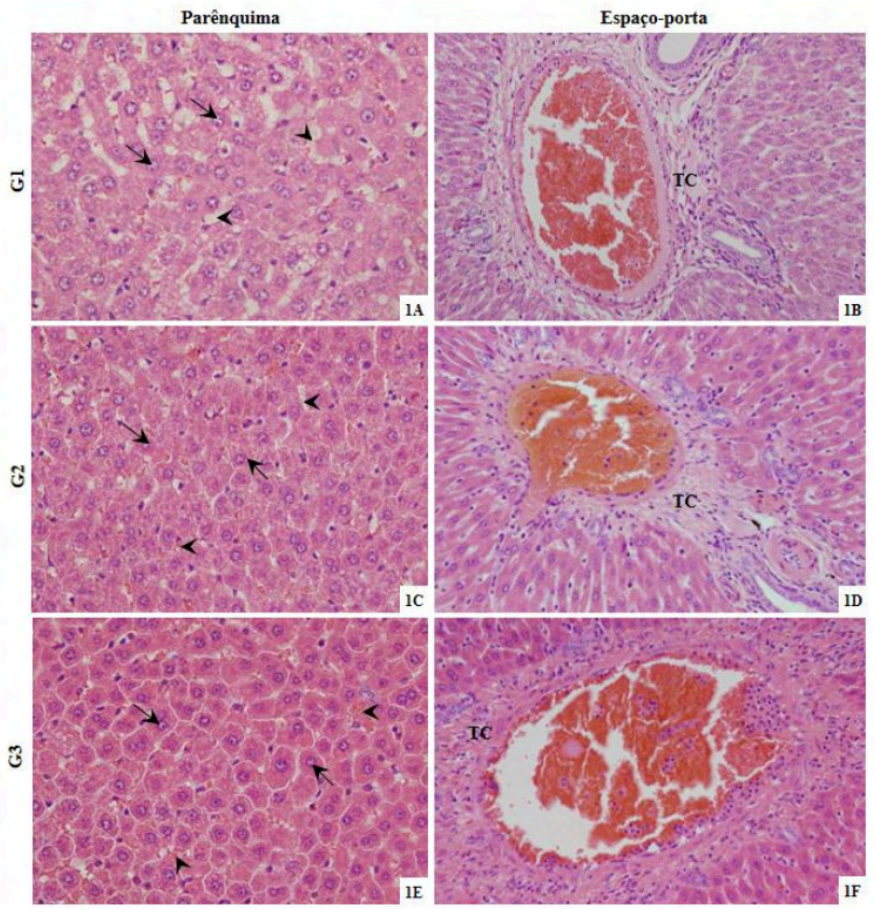
of collagen fibers was measured according to the methodology described in MANNI et al. (2001) and KOSHIMIZU et al (2013). The values obtained correspond to the percentage of birefringent collagen present in the evaluated liver area. The mean and standard deviation were calculated from the results obtained.

In the analysis of collagen-related data, statistical analysis was performed using the GraphPad Prism 6.01 program (GraphPad Software, La Jolla, CA, USA), the data obtained (collagen content) were submitted to one-way analysis of variance (One- way ANOVA) and Tukey's post-test, with an accepted significance level of 0.05.

RESULTS

MORPHOLOGICAL RESULTS

The morphological analysis of the liver of young, adult and old rats showed, under light microscopy, normal morphological characteristics of the lobular parenchyma of the liver, considering the hepatocytes, sinusoid capillaries, collagen fibers and blood vessels (Figures 1A,1C,1E). However, in some animals of the three groups the presence of inflammatory infiltrate with mild intensity was observed in the portal space region (Figures 1B, 1D, 1F). This change may be related to external factors such as keeping the animals in the vivarium or handling them.



Figures 1 A-F – Photomicrographs of liver sections from rats of groups G1, G2 and G3 stained by HE. In figures 1A,1C,1E lobular parenchyma with normal appearance: hepatocytes, (arrows) and sinusoid capillaries, (arrowheads). 695x. In figures 1B,1D,1F portal space region showing inflammatory infiltrate in connective tissue. (TC). 345x.

MORPHOMETRIC RESULTS

Nucleus of hepatocytes, sinusoid capillaries and others

The morphometric analysis did not reveal significant differences between the three groups analyzed in the frequency of hepatocyte nuclei, as well as in the frequency of sinusoid capillaries. However, a significant difference was observed in the parameter "others" between G1 and G2, however there were no differences between G1 and G3, and G2 and G3. (Figure 2)

Statistical analysis of the general structure of the liver of rats from groups G1, G2 and G3.

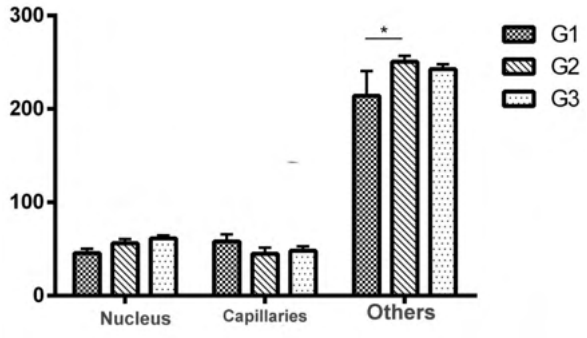


Figure 2 – Mean frequency of hepatocyte nuclei, sinusoid capillaries and others, in groups G1 (Young), G2 (Adult) and G3 (Elderly). *statistically significant difference. Tukey test ($p \leq 0.05$).

Histochemical analysis of glycogen

The study was carried out under light microscopy, with special attention to the glycogen component, its distribution and its relationship with part of the hepatic functional unit using the PAS technique, where the presence of glycogen was observed in groups G1, G2 and G3. Figures 3A, 3B and 3C.

ANALYSIS OF COLLAGEN CONTENT IN THE LIVER

The sections submitted to picrosirius staining and analyzed under a polarized light microscope made it possible to identify with greater clarity the organization of collagen fibers in the liver matrix.

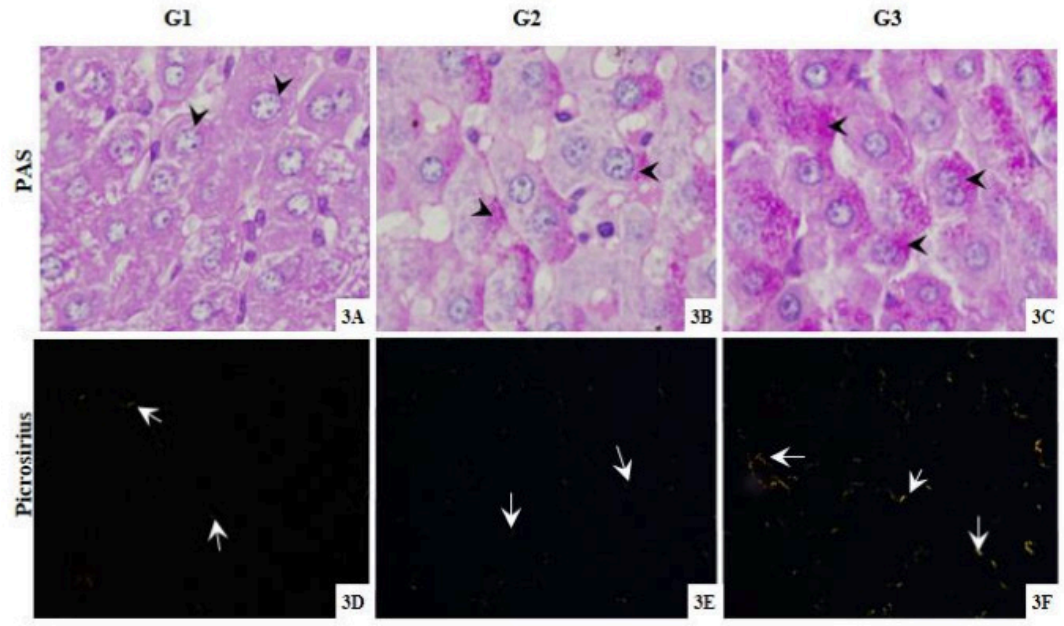
The animals from G1 (Figure 3D) had few birefringent collagen fibers in the tissue architecture, and it was possible to observe small clusters of birefringent fibers, especially in the vicinity of the portal system and central veins. In the distant regions of the portal system, large anisotropic (black) areas with small bundles of isotropic collagen fibers were observed. Statistically, G1 had the lowest amounts of birefringent collagen fibers (Figure 4).

In G2 (Figure 3E) it was possible to observe an apparent, but not significant, increase in birefringent collagen fibers. It was visible the presence of small bundles of birefringent collagen that were repeated more frequently in the midst of an anisotropic background. However, the collagen fibers of the young and adult groups do not show statistical differences (Figure 4).

G3 presented bundles of birefringent collagen fibers with greater apparent frequency, as well as greater thickness (Figure 3F). The presence of regions containing yellow/green fiber bundles is evident in all locations of the liver. In locations close to portal spaces or central veins, the presence of thick bundles becomes more evident, however, the presence of bundles of birefringent fibers is evident even in distant regions. G3 animals showed higher values in the amount of birefringent collagen fibers, being significantly higher than G1 and G2 (Figure 4).

DISCUSSION

Although aging is characterized by its degenerative aspects, which may compromise adaptation to the environment and maintenance of homeostasis (TIETZ ET AL., 1992; JECKEL-NETO E CUNHA, 2011), natural aging, or senescence must have its study directed to knowledge of the effects of



Figures 3A-C. Photomicrographs of liver sections stained with Periodic Acid-Schiff Reactive (PAS) of G1 (Figure 3A), G2 (Figure 3B) and G3 (Figure 3C). Positive PAS reaction (Arrow Head) Figure 3D-F - Photomicrographs of liver sections stained with picrosrirus red analyzed under polarized light microscope of G1 (Figure 3D), G2 (Figure 3E) and G3 (Figure 3F). In Figure 3D, the scarce presence of collagen fibers (arrows) can be seen, forming small clusters in the extracellular matrix. Figure 3E shows a modest increase in the presence of collagen fiber bundles (arrows), however, the formation of sparse bundles is visible. In Figure 3F, the presence of thicker collagen fiber bundles is evident and the greater occurrence of these birefringent collagen fibers is evident.345x

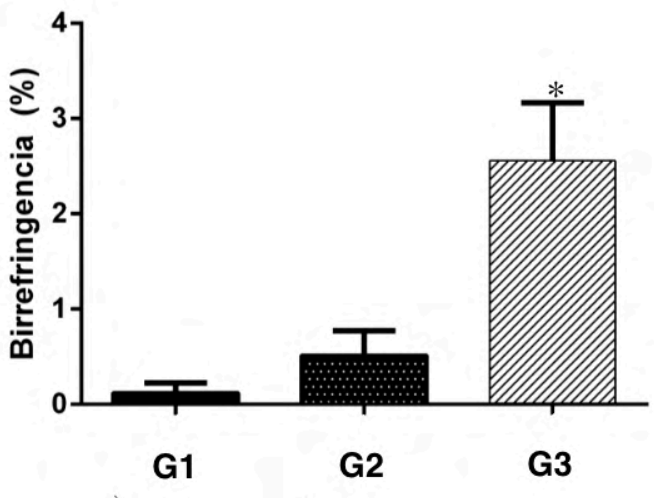


Figure 4– Graph of the quantification of birefringent collagen fibers stained with picrosirius under a polarized light microscope in the liver of G1 (Young), G2 (Adult) and G3 (Elderly). *statistically significant difference. Tukey test ($p \leq 0.05$).

aging systems, excluding associated diseases. The animals studied were naturally aged in good conditions and only two animals from the elderly group (G3) were excluded due to pathologies, in this case wounds.

The groups of rats studied had 120 days, considered young, 180 days, adults and 540 days, considered elderly and in agreement with ANDREOLLO et al. (2013), differences in anatomy, physiology, development, and biological phenomena must be taken into account when analyzing the results of any research on rats in which age is a crucial factor. Just for the purpose of better understanding the methodology and the ages studied, the second group consisted of female rats already in the reproductive phase and the third group entering menopause, avoiding making inferences in years, which vary in the literature. Thus, considering the limitations of this study, it is not intended to infer the results for the human organism and the corresponding ages, only to better understand the morphological behavior of the parameters determined by the methodology of the liver of Holtzman rats against the senescence process for the established period.

The liver is one of Organs most studied organs in aging in terms of metabolic aspects and structural changes, but the relevant literature shows that aging changes its biochemical reactions and cellular behavior in general (FAUSTO et al., 2006; MICHALOPOULOS, 2007; FERRIOLI et al., 2011; ENKHBOLD et al., 2015; SERRA et al., 2015; FERNÁNDEZ-GARCIA et al., 2018).

Being an organ of crucial importance and high regenerative capacity, it is well described morphologically by the relevant literature, including rats, thus allowing comparative analyzes (WEIBEL ET AL, 1969; GERBER, THUNG, 1987; BOGLIOLO, 1994; HUANG, LIAW, 1995; ENGELMAN et al, 2001; JUNQUEIRA, CARNEIRO, 2013).

The morphological analysis of the liver of young, adult and old rats, showed, under the light microscope, normal morphological characteristics of the lobular parenchyma of the liver, considering the hepatocytes, sinusoid capillaries, collagen fibers and blood vessels, although some animals from the three groups showed presence inflammatory infiltrate with mild intensity in the portal space region. This change may have been due to external factors such as keeping the animals in the vivarium or handling them.

Likewise, the morphometric analysis did not reveal significant differences between the three groups analyzed in the frequency of hepatocyte nuclei, as well as in the frequency of sinusoid capillaries. However, a significant difference was observed in the parameter "others" between G1 and G2, but without differences between G1 and G3, and G2 and G3.

Thus, although aging affects the organs, tissues and cell types of the same organism in different ways, resulting in differential rates of function decline, the analyzed livers aged morphologically and morphometrically under functionally adequate conditions. Such results partially agree with TIETZ et al. (1992) regarding the fact that although the liver is not free from age-related morphological changes, its homeostatic functions are not seriously compromised and liver function remains in senescent individuals, but for XAVIER (2011), liver aging includes changes macroscopic, histological and physiological.

Glycogen represents the storage form of sugars in the animal cell, being stored naturally in the hepatocyte (SOARES FILHO et al., 2011). Thus, another analysis performed in the present study was that of the glycogen component performed under light microscopy, evaluating its distribution and its relationship with part of the hepatic functional unit by the PAS technique, where

the presence of glycogen was observed in groups G1, G2 and G3, with no significant differences between the groups.

The liver plays a central role in carbohydrate metabolism, and glycogen is an important component of the liver cell, which at electron microscopy appears in the form of coarse granules, generally located in the smooth endoplasmic reticulum zone, functioning as a deposit that Hepatocyte mobilizes when hypoglycemia occurs (MULLER, SEITZ, 1984a; MULLER, SEITZ, 1984b), in the same way glycogenic liver disease can be a complication of poorly controlled diabetes mellitus with abnormal accumulation of glycogen in hepatocytes (TORBENSON et al., 2006).

Collagen is considered the most abundant component of the extracellular matrix of many types of soft tissues, and fibrosis is a characteristic of the aging of several organs, including the heart and kidney (GAGLIANO et al. 2000; HALPER, KJAER, 2014).

Thus, to analyze the collagen content in the liver, the sections were submitted to picrosirius staining and analyzed under a polarized light microscope, which allowed to identify with greater clarity the organization of collagen fibers in the liver matrix. The G1 animals had few birefringent collagen fibers in the tissue architecture, and it was possible to observe small clusters of birefringent fibers, especially in the vicinity of the portal system and central veins. In the distant regions of the portal system, large anisotropic (black) areas with small bundles of isotropic collagen fibers were observed. Statistically, G1 had the lowest amounts of birefringent collagen fibers. In G2, it was possible to observe an apparent, but not significant, increase in birefringent collagen fibers, with the presence of small bundles of birefringent collagen, which are repeated more frequently in the midst of an anisotropic background. Despite this, the collagen fibers of the young and adult groups do not show statistical differences. However, G3 presented bundles of birefringent collagen fibers with greater apparent frequency, as well as greater thickness. In locations close to portal spaces or central veins, the presence of thick bundles becomes more evident, however, the presence of birefringent fiber bundles is notorious even in distant regions. G3 animals showed higher values in the amount of birefringent collagen fibers, being significantly higher than G1 and G2, in agreement with the study by GAGLIANO et al. (2002) that moderate fibrosis is a histological feature of liver aging.

Knowing that the liver's response to injury consists of fibrosis and age is a critical factor in this process (COLLINS et al., 2013), the results of the present study significantly demonstrate the increase in the main type of fiber constituting the extracellular matrix. of aged rats, which is collagen, a condition that would be extremely potentiated by external factors, such as alcohol, affecting the homeostatic balance (FINCH, 1993; GAGLIANO et al., 2002; JOHNSON et al., 2006, JECKEL-NETO, CUNHA, 2011), an extremely important factor due to the importance of the liver in the balance of the endocrine system (LIBERMAN, 2011). Therefore, considering the results of the present study and despite the limitations imposed by the methodology applied, the liver seemed to age well in Holtzman rats without significant morphometric changes but with a more pronounced fibrosis with aging time.

CONCLUSION

The results obtained allowed us to conclude that the most relevant alterations obtained during the senescence process of the rats' liver referred to the more accentuated presence of collagen fibers.

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