EFFECT OF MELATONIN ON THE LIVER HISTOPHYSIOLOGY OF ADOLESCENT RATS EXPOSED TO ALCOHOL

ORIGINAL ARTICLE

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ABSTRACT

The present study evaluated whether melatonin administered during adolescence can prevent the deleterious effects produced by alcohol on the liver. Thirty albino female rats (*Rattus norvegicus albinus*), 40 days old, virgins, weighing approximately 150±10g, from the Wistar lineage and were divided into the following groups: I – Adolescent rats that did not receive alcohol and euthanized at 60 days of life; II - Adolescent rats subjected to chronic alcohol consumption and euthanized at 60 days of life; III - Adolescent rats subjected to chronic alcohol consumption and simultaneously treated with melatonin, and euthanized at 60 days of life. The collected organs underwent histological processing and to obtain the results, morphometric, histopathological and histochemical analyzes were performed. Alcohol was administered by gavage. intragastric injection at a dosage of 3 g/Kg of ethyl alcohol in the rats of groups II and III for 20 days. Melatonin was administered in daily injections of 0.8 mg/Kg, always at the beginning of the night, intraperitoneally for 20 days. When analyzing the weight of the animals, no significant difference was observed between the groups. The animals in group II presented alterations in the livers such as congestion of the centrilobular vein, hepatocellular ballooning,

microgoticular steatosis, leukocyte infiltrate, several pyknotic nuclei in the livers, increase in lobular parenchyma and reduction in non-lobular parenchyma, greater deposition of collagen and reduction of glycogen. Melatonin treatment prevented all these changes. With this, we can conclude that melatonin has great therapeutic potential in the prevention of liver damage in adolescent rats subjected to moderate alcohol consumption, in addition to positive effects on the deposition of collagen and glycogen in the liver.

Keywords: Teenagers, Alcohol, Antioxidant, Liver, Inflammation.

1. INTRODUCTION

The consumption of alcoholic beverages is common worldwide, and factors such as age, culture, gender and religion influence both the frequency and the volume consumed. About 43% of the global population consumes alcoholic beverages, in the Americas region, this index is higher than the global one, reaching 54% (WHO, 2018). In Brazil, approximately 25 million inhabitants, which is equivalent to 16.5% of the population, consumed more alcohol in 2015 (FIOCRUZ, 2017). According to Andrade; Siu (2019), in 2016, the Americas region was the third in the world in relation to excessive alcohol consumption by young people, registering a percentage of 18.5%, behind only the West Pacific (18.8%) and Europe (24.1%). In Brazil, young people aged 18 to 24 years are in second place in the proportion of alcohol consumption, corresponding to 35.1%, second only to individuals aged 35 to 34 years (38.1%) (FIOCRUZ, 2017). According to WHO (2018), in 2016, the prevalence of women who consumed alcohol was 32.3%, lower than that of men, which was 53.6%. Despite the indices pointing to lower consumption of alcoholic beverages by women, they are more affected, as they are biologically more vulnerable to the effects of alcohol than men. Thus, they are more likely to develop alcohol-related problems even with lower consumption levels and/or at an earlier age than men (ANDRADE; SIU, 2019).

EtOH is a small molecule, uncharged and soluble in water, and for this reason it can easily cross cell membranes (NORBERG *et al.*, 2003; VONGHIA *et al.*, 2008). Ingested alcohol is metabolized by the liver, where the largest amount of

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metabolizing enzymes is present (MINCIS; *et al*, 2011). After its absorption, EtOH is converted by Alcohol Dehydrogenase (ADH), catalase or cytochrome P450 2E1 (CYP2E1) into acetaldehyde. After this step, Aldehyde Dehydrogenase (ALDH) converts acetaldehyde into acetate (BEST; LAPOSATA, 2003). These reactions promote increased respiratory chain activity, and consequently increased ROS production (BROCARDO *et al.*, 2011). Alcohol products have been considered hepatotoxins that act directly or indirectly on the organ (ROCCO *et al.,* 2014), in addition to causing necrosis or apoptosis of hepatocytes (FARKAS; KEMÉNY, 2013).

As the liver is the main organ responsible for metabolizing alcohol, it is the one that suffers most from the harmful effects of this xenobiotic (ROCCO *et al.,* 2014). Continuous exposure to EtOH leads to the progression of fat accumulation in the liver, steatosis, to the inflamed state, steatohepatitis (YANG *et al.*, 2010). Steatosis is the initial alcoholic disease, later subsequent inflammation and alcoholic hepatitis may occur, resulting in liver fibrosis (FARKAS; KEMÉNY, 2013).

Melatonin, a hormone produced mainly by the pineal gland, with the ability to perform numerous receptor-dependent and independent mediated actions, may act in the control of the circadian cycle, promoting sleep and performing an antioxidant action, reducing oxidative stress, in addition to having anti- inflammatory and anti- apoptotic drugs (HARDELAND; PANDI-PERUMAL; CARDINALI, 2006; MAHIEU *et al.*, 2009; REITER; TAN; GALANO, 2014). Its antioxidant capacity was discovered in 1993 (POEGGELER *et al.*, 1993), and has been used in the prevention of diseases caused by oxidative stress, due to its ability to eliminate free radicals and improve antioxidant levels (NWOZO; AJAGBE; OYINLOYE, 2012). This indolamine and its metabolites act in the direct elimination of several free radicals, such as the hydroxyl radical (OH), peroxynitrite (ONOO), nitric oxide (NO) and O2 radical, in addition to the reactive species of oxygen, nitrogen and sulfur (AMARAL *et al.*, 2019; GALANO; TAN; REITER *et al.*, 2018; GUNATA; PARLAKPINAR; ACET, 2020).

With regard to the liver, some studies with rats subjected to alcohol consumption have already demonstrated positive aspects of melatonin administration on the liver structure (KURCER *et al.*, 2007; KURHALUK; TKACHENKO; LUKASH, 2020), on the activity of antioxidant enzymes (KURCER *et al.*, 2007), inhibition of pro-inflammatory cytokines (HU *et al.*, 2019) and apoptosis (ULLAH *et al.*, 2020). Thus, this indolamine has great potential to prevent changes caused by alcohol. With this, this work aimed to evaluate whether melatonin administered to adolescent rats could prevent the deleterious effects produced by alcohol in the liver.

2. MATERIAL AND METHODS

2.1 ANIMAL ACQUISITION

Thirty albino female rats (*Rattus norvegicus albinus*), 40 days old, virgins, weighing approximately 130±10g, of the Wistar lineage, coming from the Vivarium of the Department of Animal Morphology and Physiology (DMFA), of the Federal Rural University of Pernambuco (UFRPE) were used. The animals were confined in cages and maintained with food and water *ad libitum,* with a temperature of 22±1ºC and artificial lighting produced by fluorescent lamps (Phillips brand, daylight model, 40W), with a 12-hour photoperiod. light and dark for 12 hours, considering the light period from 6:00 am to 6:00 pm. After an adaptation period of seven days, vaginal smears were taken to determine the estrous cycle. Females that had three consecutive regular estrous cycles were selected for the experiment, which consisted of the following experimental groups with 10 animals each:

Control – Adolescent rats that did not receive alcohol and euthanized at 60 days of age;

Alcohol – Adolescent rats subjected to chronic alcohol consumption and euthanized at 60 days of life;

Alcohol + Melatonin – Adolescent rats subjected to chronic alcohol consumption and simultaneously treated with melatonin and euthanized at 60 days of life.

The experiments were approved by the institutional ethics committee under license CEUA/UFRPE Nº 041/2019.

2.2 ETHANOL ADMINISTRATION

It was administered by gavage intragastric injection at a dosage of 3 g/kg of ethyl alcohol in the rats of groups II and III for 20 days (ARAÚJO-FILHO *et al.*, 2007; MARCO *et al.*, 2017; SCHEIDT *et al.*, 2015; VARLINSKAYA; SPEAR; SPEAR, 2001; VEIGA *et al.*, 2007).

2.3 MELATONIN TREATMENT

Melatonin, N-acetyl-5-methoxytryptamine (Sigma Chemical Co., St. Louis, USA) was administered in daily injections of 0.8 mg/kg, for a period of 20 days. For this, melatonin was dissolved in 0.2 mL of ethanol and diluted in 0.8 mL 0.9% NaCl. Injections were applied intraperitoneally, between 6:00 pm and 7:00 pm. This dose is comparable to the human dose (9 mg/kg), which was converted based on body surface area (ABD-ALLAH *et al.*, 2003; MOUSTAFA *et al.*, 1999; PAGET; BARNE, 1994). The animals in groups I and II received the hormone vehicle.

2.4 WEIGHT OF ANIMALS

The rats were weighed daily throughout the experimental period and for statistical analysis days 1, 10 and 20 were considered.

2.5 HISTOPATHOLOGY AND HISTOCHEMISTRY OF THE LIVER

For liver collection, the rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (6.0 mg/kg) intramuscularly. Then, the abdominal cavity was

opened to remove the organ. The rats were euthanized using anesthesia deepening with ketamine hydrochloride (80mg/Kg) and xylazine (6mg/Kg), associated with thiopental (100 mg/kg), intraperitoneally. Liver fragments were immersed in buffered formalin at 10% and pH 7.4, remaining there for 48 hours. After these procedures, they were dehydrated in ethyl alcohol (increasing concentrations), cleared with xylol, impregnated and included in paraffin. The paraffin blocks were cut in a Minot type microtome (Leica RM 2035) adjusted to 5 μm. The sections obtained in this way were placed on glass slides and taken to an oven regulated at a temperature of 37°C for 24 hours, for drying and bonding. In sequence, the sections were stained with hematoxylin-eosin (HE), picrosirius and PAS (Periodic Acid Schiff) and analyzed in an OLYMPUS BX-49 light microscope and photographed in an OLYMPUS BX-50 microscope.

2.6 MORPHOMETRY

Morphometric study was carried out according to the methodology described by Engelman *et al.* (2001). The proportion between the non-lobular and lobular parenchyma of the liver of the rats in the experimental groups was determined by stereological methods, using a grid with 100 test points, placed on the sections of the histological preparations stained with Mallory's trichrome, as this facilitates visualization. of the portal spaces when using stereological methods. The counting was done on three slides, so that 10 fields were counted using the 40x objective, making a total of 3,000 points per group.

A semi-quantitative assessment of liver change scores proposed by the NASH Committee on Pathology (Nonalcoholic Steatohepatitis) Clinical Research Network (KLEINER *et al.*, 2005), which are required to follow: steatosis (<5% = 0; 5– 33% = 1; 33–66% = 2; > 66% = 3); lobular inflammation (none = 0; 2 foci = 1; 2-4 foci = 2; > 4 foci = 3); and hepatocellular ballooning (none = 0; few = 1; prominent = 2). All features were blindly scored on three slides/group, evaluated five fields/slide. For this, the images were captured with a Sony® video camera coupled to an Olympus®

Bx50 microscope. All quantifications were performed using the Gimp®2.8 image editor.

2.7 STATISTICAL ANALYSIS

To compare the weight of the rats, morphometry and triage and histochemistry data, the Analysis of Variance was performed, when significant, it was complemented by the Multiple Comparisons test by Tukey and Kramer. A significance level of 0.05 ($P < 0.05$) was adopted.

3. RESULTS

3.1 WEIGHT OF ANIMALS

There was no significant difference between the weights of rats treated with alcohol, alcohol + melatonin when compared to the control group on days 1, 10 and 20 (Table 1).

Table 1: Mean ± standard deviation of the weight of the rats in the experimental groups, on days 1, 10 and 20

| Treatment	Weight (g)		
	1 day	10 days	20 days
Control	123.60 ± 3.78 a	139.60 ± 5.87 a	158.40 ± 5.24 a
Alcohol	126.50 ± 4.17 a	139.30 ± 5.23 a	157.40 ± 4.23 a
Alcohol + Melatonin	125.90 ± 1.52 a	135.80 ± 2.14 a	156.80 ± 2.98 a
	F=0.21 P=0.8145	F=0.20 P=0.8185	F=0.04 P=0.9646

Source: Authors, 2023.

1 Means followed by the same letter do not differ according to the Tukey and Kramer test (P<0.05).

3.2 HISTOPATHOLOGICAL ANALYSIS OF THE LIVER

Histopathological analysis of the liver of females in the control and alcohol + melatonin groups revealed unchanged hepatic parenchyma with organized hepatocyte cords surrounding the central lobular vein, interspersed by sinusoid capillaries. However, in the livers of rats in the alcohol group, congestion of the centrilobular vein, hepatocellular ballooning in the areas around the centrilobular veins, microgoticular steatosis, leukocyte infiltrate, and several hepatocytes with pyknotic nuclei were observed (Fig. 1).

Figure 1. Photomicrograph of livers of females in experimental groups. A - B (control); C–D (alcohol); E - F (alcohol + melatonin). Central lobular vein without congestion (long arrow); Central lobular vein with congestion (Vcc); Hepatocyte ballooning (short arrows); leukocyte infiltrate (IL); Multigoticular steatosis (dashed arrows); Hepatocytes with pyknotic nuclei (arrowheads). HE
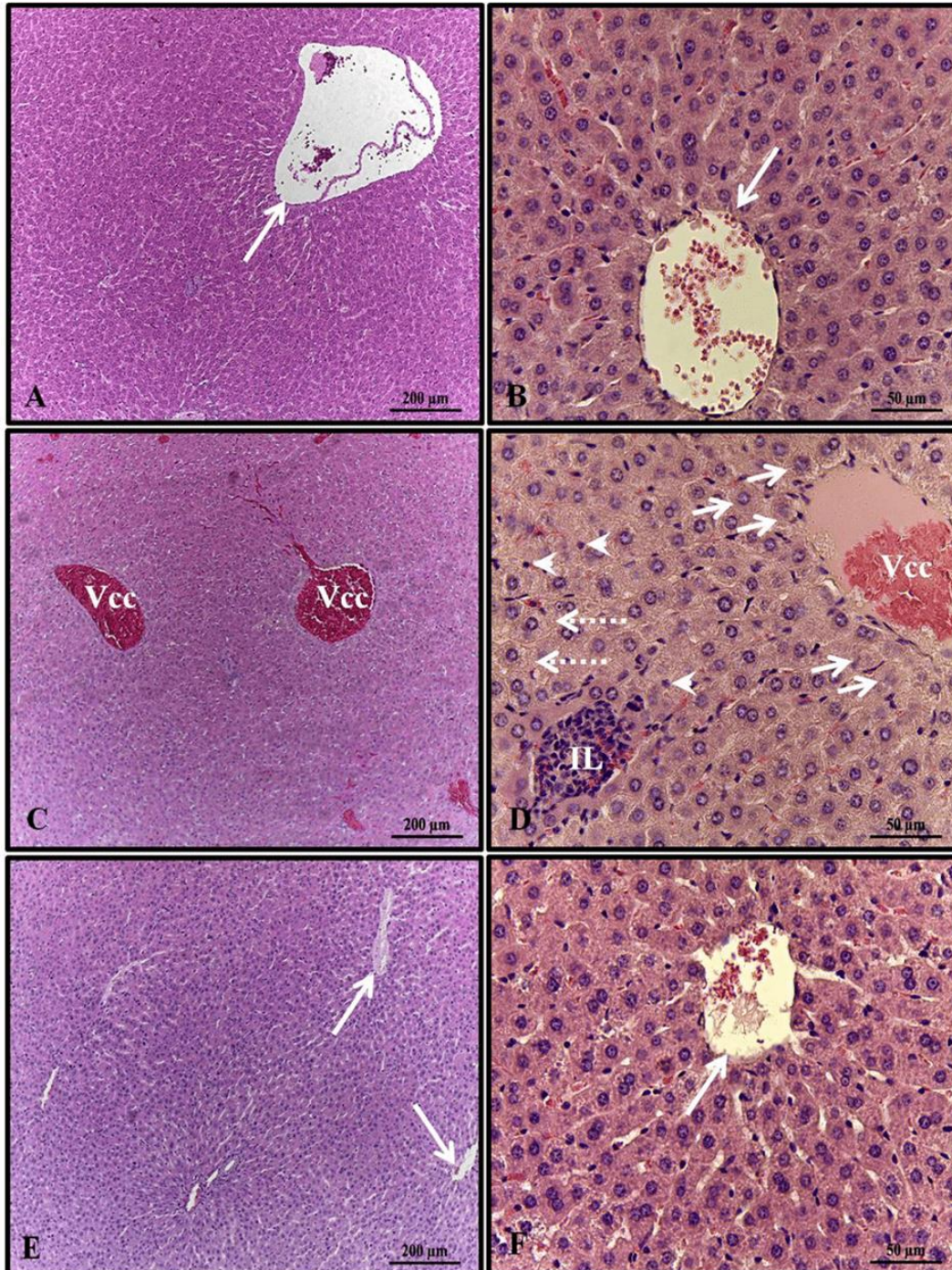
Source: Authors, 2023.

3.3 LIVER MORPHOMETRIC ANALYSIS

There was an increase in the percentage of lobular parenchyma, promoting a reduction in the non-lobular parenchyma in the liver of rats in the alcohol group. Melatonin prevented this effect, presenting similar parameters in relation to the control group (Table 2).

Table 2. Mean ± standard deviation of the percentage of lobular and non-lobular parenchyma in the liver of females in the experimental groups

| Groups	Control	Alcohol	Alcohol + Melatonin	p
lobular parenchyma	$72.56 \pm 2.19b$	$88.21 \pm 1.39a$	$73.62 \pm 2.81b$	0.0009
Parenchyma non- lobular	$24.44 \pm 1.11a$	$11.79 \pm 3.74b$	$26.38 \pm 1.93a$	0.0125

Source: Authors, 2023.

Means followed by the same letter in the lines do not differ significantly by the Tukey and Kramer test ($p < 0.05$).

3.4 LIVER ALTERATION SCORES

In the semi-quantitative evaluation of the scores of liver alterations, females in the alcohol group revealed high scores of steatosis, lobular inflammation and ballooning hepatocytes. Melatonin treatment considerably reduced these effects, with scores similar to the control group (Fig. 2).

Figure 2. Liver alteration score chart in females of experimental groups. Check for a significant increase in steatosis, lobular inflammation and hepatocellular ballooning. Means followed by the same letter do not differ significantly according to the Tukey and Kramer test (p<0.05)
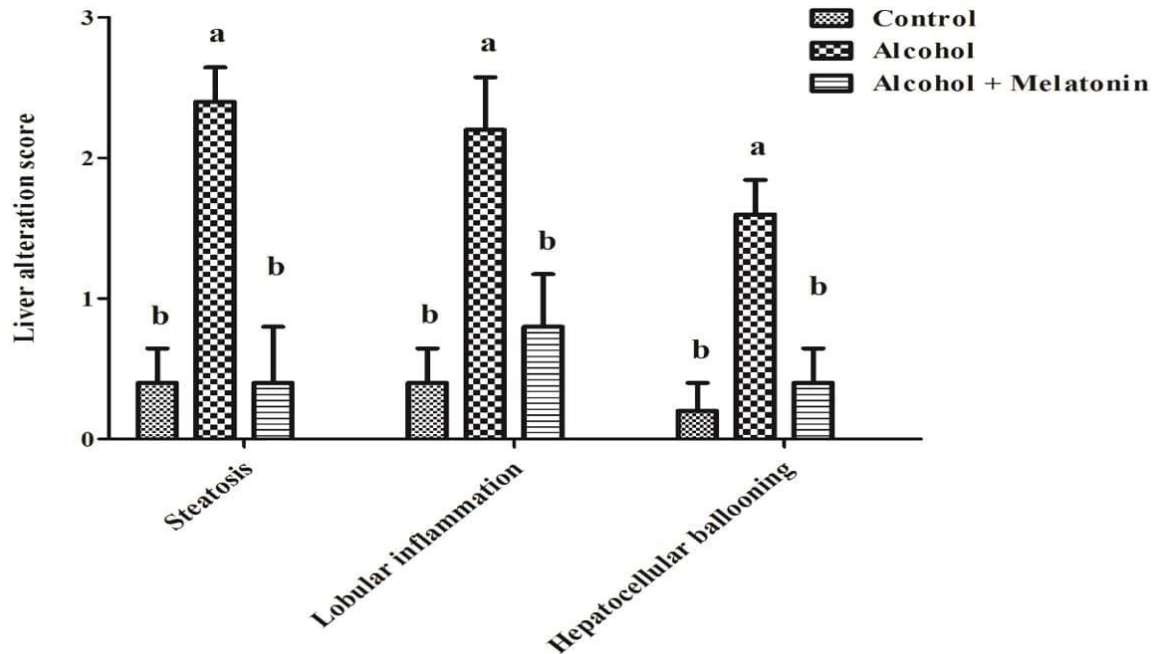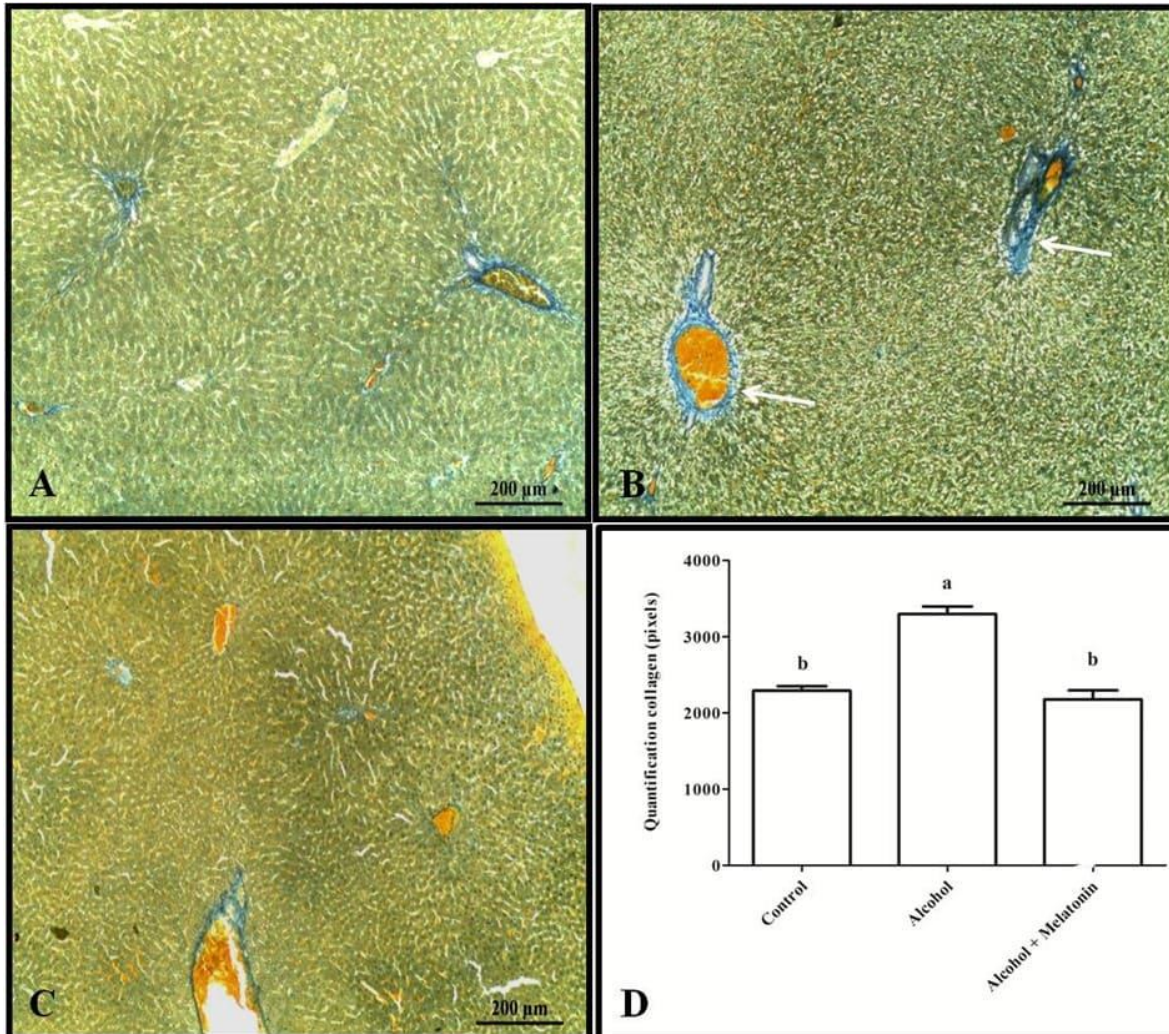

Source: Authors, 2023.

3.5 LIVER HISTOCHEMISTRY

Trichrome staining and quantification in pixels revealed increased collagen in the liver around the veins, characterizing venular fibrosis in females that received only alcohol (Fig. 3). However, with regard to liver glycogen, the animals in this group showed a significant reduction when compared to the other groups (Fig. 4).

Figure 3. Histochemistry for collagen in the liver of females in the experimental groups. A (control); B (alcohol); C (alcohol + melatonin). Note in B greater marking characterizing venular fibrosis (arrow). D - Quantification in pixels. Mallory's trichrome. Means followed by the same letter do not differ significantly according to the Tukey and Kramer test (p<0.05).

Source: Authors, 2023.

Figure 4. Histochemistry for glycogen in the liver of females in experimental groups. A (control); B (alcohol); C (alcohol + melatonin). Observe in B several less stained areas (*), signaling the absence of glycogen. D - Quantification in pixels. SBP Means followed by the same letter do not differ significantly according to the Tukey and Kramer test (p<0.05)
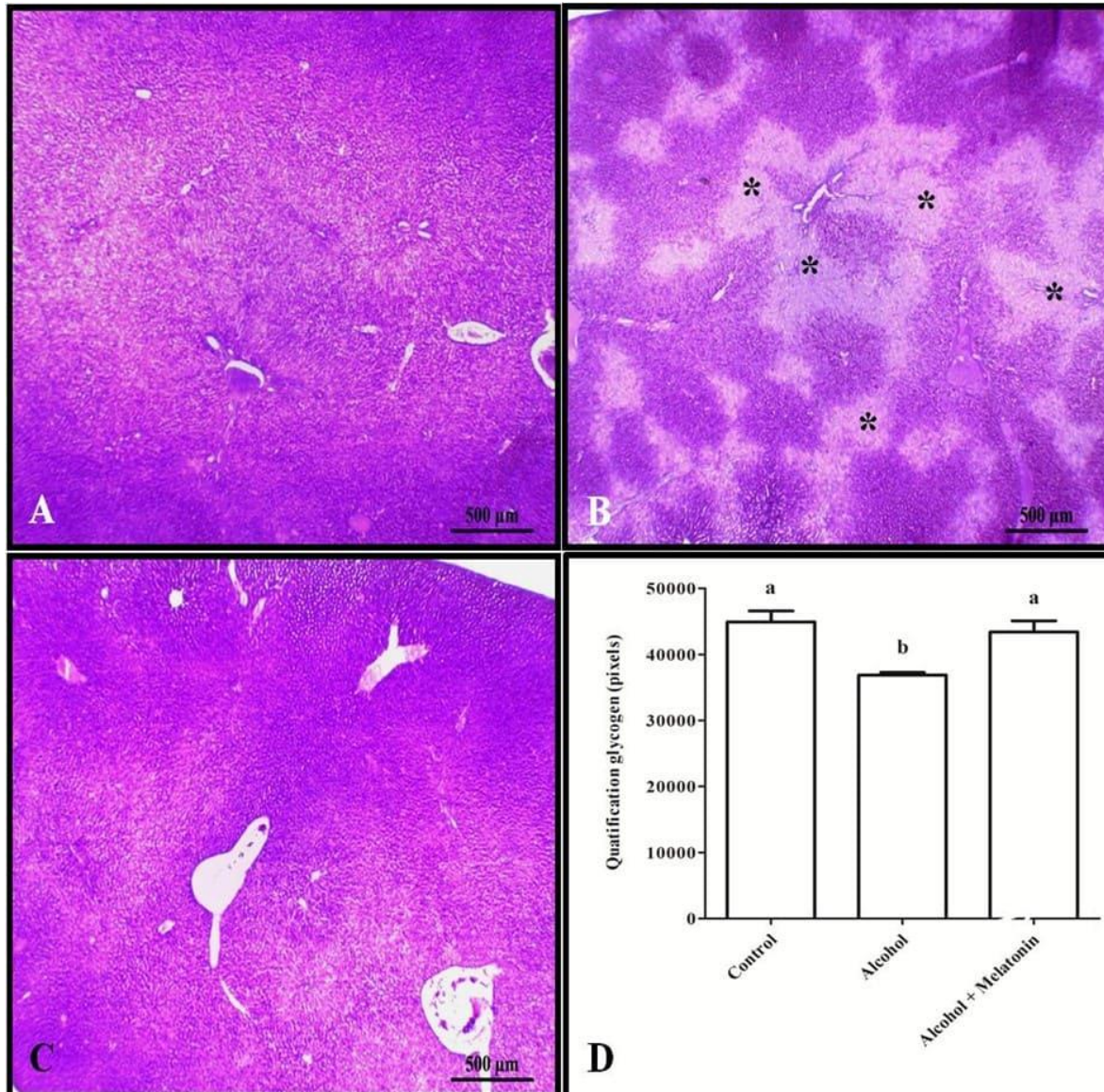
Source: Authors,2023.

4. DISCUSSION

The liver is the main organ that suffers the deleterious effects of toxic substances (BHADORIA *et al.*, 2015). Alcohol consumption causes structural changes resulting

from oxidative stress. Therefore, it is one of the central mechanisms of liver damage (RADIC *et al.*, 2019). In the present study, it was found that in the alcohol group, the liver showed congestion of the central lobular vein, corroborating the results presented by Radic *et al.* (2021), where, according to Allaithi; Alazawi (2019) can be explained by the accumulation of fat in the liver, which in turn stimulates an inflammatory response by stellate cells, thus releasing inflammatory cytokines in order to bring a greater amount of white blood cells to the affected region. What would also justify in our findings the presence of leukocyte infiltrate in the hepatic parenchyma. The presence of these infiltrates, mainly neutrophils within the hepatic parenchyma, is a characteristic indicative of alcoholic hepatitis (RAMAIAH; JAESCHKE, 2007) and occurs abundantly in the portal tracts (MOSTAFA *et al.*, 2020)*.*

According to Tao *et al.* (2021) and Yeh; Brunt (2014), steatosis and hepatocyte ballooning are among alcoholic liver injuries, which was also evidenced in our study. Steatosis is considered the most frequent form of alcoholic liver disease and precedes other alterations, such as steatohepatitis and fibrosis (KISSELEVA; BRENNER, 2019; GAO; BATALLER, 2011). It is characterized by impairment of the hepatic lobular parenchyma resulting from the accumulation of lipids in hepatocytes. This impairment is observed mainly around the central lobular vein, however, with the progression of the pathology, it can involve all areas, and is often accompanied by a chronic inflammatory infiltrate (MOSTAFA *et al.*, 2020)*,* as we could observe in our study. Hepatic ballooning, on the other hand, is the morphological modification due to hepatocellular stress resulting from the oxidation of free fatty acids, and endoplasmic reticulum stress due to fat accumulation, thus promoting injuries to the intracellular environment, such as changes in Perilipin. Damage is seen through morphological changes such as cell swelling, enlargement of hepatocytes, rounding of the cell contour, reticulation and clearing of the cytoplasm from a viscous material called Mallory-Denk bodies or mallory hyaline, which is composed of intermediate

filaments well phosphorylated, ubiquitin - binding protein P62 and ubiquitin in the cytoplasm of hepatocytes (MOSTAFA *et al.*, 2020; SCHILD; GUY, 2018).

Steatosis can progress to its inflammatory state, steatohepatitis. In later and more severe cases, the disposition of collagen resulting from parenchymal damage, such as chronic inflammation, necrosis and subsequent cell regeneration, can cause fibrosis in response to exacerbated regenerative activity (MASSEY; ARTEEL, 2012; ROY *et al.*, 2015), which justifies the increase in collagen in the animals in the Alcohol group. It was also verified in our findings, hepatocytes with pyknotic nucleus. According to the literature, this is indicative of low cell activity, suggestive of a cell necrosis process (BHADORIA *et al.*, 2015).

In our research, the ingestion of EtOH also caused a marked decrease in the glycogen reserve of the hepatic parenchymal tissue. In the liver, glucose is transported to hepatocytes by means of transport proteins that are present in its plasma membrane (NANJI; FOGT; GRINIUVIENE 1995), thus promoting the formation of the sugar reserve in the form of glycogen through Glycogen Synthase (GS). The consumption of EtOH does not promote changes in the phosphorylase activity of GS, however, it promotes a reduction in expression levels and in its mRNA (MARTIN *et al.*, 2004; SRINIVASAN; SHAWKY; KAPHALIA, 2019), which causes a decrease in the glycidic reserve total in periportal and perivenous hepatocytes (STEINER; CROWELL; LANG, 2015).

Alcohol+melatonin group showed attenuation of the structural changes found in the liver of animals in the alcohol group, with parameters close to those of the control group. It has already been shown that this indolamine is a splendid non-enzymatic antioxidant (SINGH; JADHAV, 2014), with a high capacity to minimize changes and help maintain the usual arrangement of hepatocytes (GHOSH *et al.*, 2023*)*, in addition to being widely distributed in the body. This is due to its structure having electron donor sites on carbon 2 and 3 of the pyrrole ring, and its amphiphilic nature, that is, lipophilic and hydrophilic, thus providing the ability to easily pass cell

membranes and blood-brain barriers (REITER *et al.*, 2013), thereby reducing oxidative stress by scavenging free radicals of all kinds (REITER *et al.*, 2014). Therefore, melatonin may have acted on abnormalities, preventing imbalance in antioxidant and pro-oxidant mechanisms, which in turn may have delayed or prevented the development of lesions resulting from oxidative stress (HU *et al.*, 2009) *in* the alcohol + group. melatonin.

According to Galano, Tan and Reiter (2011), this indolamine has the ability to promote a reduction in lipid peroxidation and an increase in the concentration of antioxidants by modulating their expression, in addition to the potential to prevent the reduction of glutathione (GSH) when used over a prolonged period, in addition to decreasing and the sensitization of kupffer cells to alcohol, which reduces ROS levels. As a result of this reduction, it indirectly acts on leukocyte infiltrates, promoting an improvement in inflammation (ZHANG *et al.*, 2021). However, melatonin also acts directly on inflammation, as it has the ability to attenuate the migration of neutrophil infiltrates in response to injury (KURHALUK; TKACHENKO 2020). It has already been demonstrated by Ghosh *et al.* (2023) in an experimental model of oxidative stress in rats, that this indoleamine acts to reduce collagen deposition in the liver, thus preventing fibrosis through and acting to reduce fibroblast proliferation (LI *et al.,* 2020), thus promoting an indirect response in fibrosis by cytokines. As for the reserve of sugar in the liver, the alcohol + melatonin group presented a glycogen reserve close to the control group. Previous studies have pointed out that melatonin has the ability to increase hepatic glycogen content (FARIA *et al.,* 2022), and this increase can be explained by the improvement in tissue glucose uptake and/or insulin sensitivity through a dependent pathway. of Akt activated via melatonin receptor (SHIEH *et al.*, 2009). Thus, it can prevent the reduction of glycogen reserves observed in the group subjected to chronic alcohol consumption.

5. CONCLUSIONS

The results of the present study demonstrated that melatonin has great therapeutic potential in preventing damage to the liver of adolescent rats subjected to immoderate alcohol consumption, in addition to positive effects on the deposition of collagen and glycogen in the liver. However, it is necessary to deepen studies in this area to obtain more data that prove and reinforce the potential of this hormone in the preventive therapy of alterations and pathologies caused by chronic alcohol consumption.

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