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Original Article

Effects of early and late adverse experiences on morphological characteristics of Sprague-Dawley rat liver subjected to stress during adulthood

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Abstract: The literature indicates that early rupture of the maternal bond and social isolation are variables involved in social and emotional behaviors and in increase in anxiety, particularly in stressful situations. The liver plays a role in the adaptation to stress, yet the possible morphologic changes that its structure can suffer have been studied very little. Therefore, the aim here was to ascertain, through the model of altering the early mother-infant bond and the late social bond through isolation, the effect on the stereologic characteristics of the liver in adult Sprague-Dawley rats subjected to intermittent chronic stress. Twenty-five newborn female rats were used, distributed into 5 groups, under standardized lactation and feeding conditions. The experimental groups were exposed to early (E1), late (E2), and early-late (E3) adverse experiences and then subjected to intermittent chronic stress in adulthood. The liver of each animal was isolated, and the stereologic characteristics of Nv, Vv, and Sv of the hepatocytes were determined. The results from the experimental groups were significantly higher than those obtained in the control groups. The highest values were found in group E3 (Nv = 4.43 ± 0.89 x 10^5/mm^3, Vv = 68.74 ± 2.01%, Sv = 68.78 ± 3.77 mm^2/mm^3). Considering these results, the hepatic morphology can be affected by exposure to chronic stress; however, when the individuals have been subjected to previous adverse experiences, the changes are more evident.

Keywords: Adverse experience, stress, liver, rat, morphology

Introduction

The concept of stress refers to the nonspecific response of the body to any external or internal, real or perceived force that triggers "General Adaptation Syndrome" in the body, stimulating the pathways in the brain that lead to the activation of the hypothalamo-pituitary-adrenal (HPA) axis and the central sympathetic system [1, 2]. The liver plays a fundamental role in the adaptation of the neuroendocrine response to stress when its anabolic activity provides the energy-rich compounds, such as glucose and lipids, needed so the body can adapt [3]. The adaptive response of the liver is triggered by the increased glucocorticoids in the serum induced by stress, which among other actions stimulates the liver to gluconeogenesis [4].

Several studies have reported that the stress response might damage the liver, likely due to misalignments in the HPA axis and therefore in the plasma levels of the glucocorticoids. Youssef *et al.* [5] assessed the relationship between anxiety and depression and the severity of histologic characteristics in patients with nonalcoholic fatty liver disease; they found that patients with clinical depression were 3.6 times more likely to have severe hydropic degeneration than patients without depressive symptoms. By contrast, the literature also includes studies that report various results in the plasma corticosterone levels and their relation with behavior based on animal models, mainly rodents, in which different types of stress (physical or social) and exposure time (acute or chronic) were applied [6-11]. However, it has yet

to be better understood which mechanisms are involved in the interaction between the alteration of the stress-induced plasma levels of the glucocorticoids and their effects on hepatic morphology and function.

Acute stress, emotional or social, stimulates the degradation of hepatic glycogen, presenting with or without hyperglycemia, depending on the particular characteristics of the stressor [12], with loss of hepatocyte integrity, reflected in the increase in SGOT and SGPT enzymes [13]. The aggressive encounter in the intruder-resident model produces a hepatic lesion that is also reflected by the presence of leukocytes infiltrating the hepatic parenchyma in response to the production of inflammatory mediators [14].

In terms of chronic psychosocial stress, Djordjevic *et al.* [15] proposed that the antioxidant capacity of the enzymes in the liver may be permanently altered and thus jeopardize its ability to create an adaptive response to a subsequent stressful event. To better understand the mechanisms involved in the stress response, the authors used adult Wistar rats exposed to chronic stress by isolation for 21 days. The results showed a decrease in plasma corticosterone and glucose levels, together with a significant increase in superoxide dismutase in the liver. Furthermore, there was compromise during restoration of the redox balance due to irreversible alterations in antioxidant enzymes. McIntosh *et al.* [16] showed that altered GC levels can produce toxicity by generating hydrogen peroxide through the increase in the base level of reactive oxygen species produced by the hepatic cells through the increased superoxide dismutase and the decreased antioxidant capacity resulting from the low activity of catalase and glutathione peroxidase. Consistent with these results, Kumar *et al.* [17] reported a close relationship between intermittent chronic stress induced by the administration of adrenalin and the degeneration of the nucleus and cytoplasm of the hepatic cells. They also observed that hepatocyte degeneration and an increase in SGOT result in hepatotoxicity.

Stress can also have a double effect (reducing or enhancing) on the inflammatory process that occurs in the liver. Thus, the glucocorticoids released during activation of the HPA axis have been shown to be intimately involved in the control of the inflammatory response in the liver [18]. Glucocorticoids inhibit the production of inflammatory mediators by stellate macrophages, there being a differential action through the suppression of the synthesis of the pro-inflammatory cytokines IL-1, IL-6, and TNF-α. By contrast, Liao *et al.* [19] showed that perfusion of corticosterone *in situ* in the liver within physiologic ranges induces the production of IL-6 and TNF-α by stellate macrophages, Czech *et al.* [20] reported that chronic psychosocial stress in mice, produces high levels of TNF-α in the liver, induces oxidative stress and liver inflammation, suggesting that it is not the entire effect of the GCs on the production of pro-inflammatory cytokines that is suppressive.

Other studies have provided evidence of possible morphoquantitative changes in the liver as a result of stress. Sharma [21] and, more recently, Agrawal and Gupta [22] showed that hepatocyte number density (Nv) and volume density (Vv) increase as the frequency of exposure to heat stress increases. Thus, Sharma [20] suggested an association between heat-induced intermittent chronic stress and accelerated hepatocyte proliferation, whereas Agrawal and Gupta [22] concluded that the increasing degenerative and necrotic changes in the hepatic parenchyma confirm the regenerative power of the liver.

The importance of the effects of stress on the liver has been shown in animal models. Our aim was to determine the effects of stress experienced in early stages on the morphology of the liver in adult animals because early experiences modulate individual responses to stress and the susceptibility to disease through mechanisms that could influence the programming of the HPA axis [23-25].

The characteristics and quantity of individuals who share the same place seem to have a strong impact on the reactions to stress; therefore, social isolation used as a stress factor could cause damage to the liver, similarly to that previously described for animal models. Studies have indicated that social isolation in rats generates great anxiety and aggression in the intruder-resident model [26] and exerts influence on the hormones of the HPA axis with an increase in the plasma corticosterone levels [9]. More recently, Vásquez *et al.* [27] showed that social isolation in rats was able to produce morphologic changes in the adrenal gland, altering stereologic parameters, such as hepatocyte number density, percentage of glandular

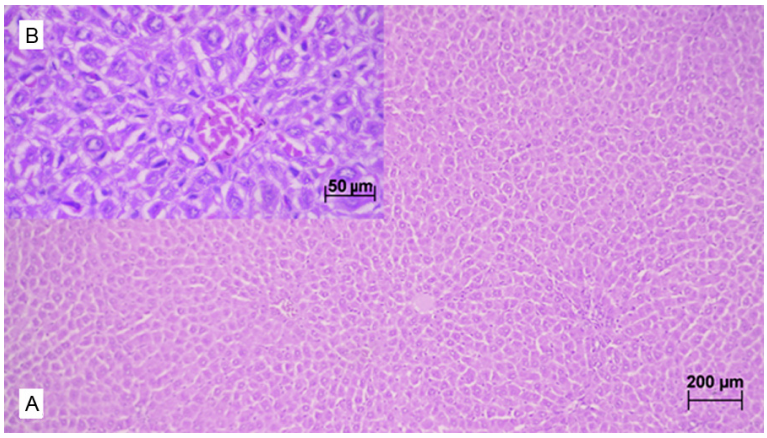

Figure 1. Liver of Sprague-Dawley rat, C1 group. A. Preserved lobular architecture is observed. B. Hepatocytes and sinusoids present a normal distribution and appearance. H&E staining.

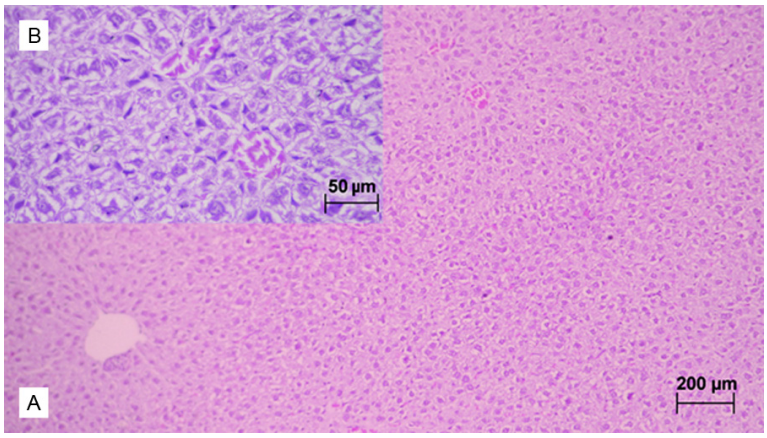

Figure 2. Liver of Sprague-Dawley rat, E1 group. A. Disorganization of the hepatic cords is observed. B. Larger hepatocytes and slight hydropic degeneration. H&E staining.

tissue, and hepatocyte surface, compared to the control group.

The aim of this study was to ascertain experimentally, through the model of altering the early mother-infant bond and the late social bond through isolation, the effect on the histologic characteristics of the liver in adult Sprague-Dawley rats subjected to intermittent chronic stress.

Materials and methods

Animals

Twenty-five Sprague-Dawley female albino newborn rats were used, taken from the Experimental Surgery Unit of the Doctorate in Morphological Sciences of the Faculty of Medicine at the Universidad de La Frontera. They were kept in controlled environmental conditions in terms of temperature, environmental noise, and a cycle of 12 hours light/12 hours darkness. The experiments were conducted according to the directives of the Guide for the Care and Use of Laboratory Animals [28]. The Ethics Committee of Universidad de La Frontera (DI11-0054) approved this project.

The rats were divided into five groups of five animals each, randomly assigned.

Experimental group 1 (E1): subjected to early adverse experience.

Experimental group 2 (E2): subjected to late adverse experience.

Experimental group 3 (E3): subjected to early-late adverse experience.

Control group 1 (C1): not subjected to early or late adverse experiences; without exposure to intermittent chronic stress.

Control group 2 (C2): not subjected to early or late adverse experiences.

Early adverse experience (E1): (alteration of the mother-infant bond through reduction of the lactation period).

Newborn rats with 18 days of lactation were separated from their mother and kept in a cage under conditions of social interaction, with water and food (pellets) *ad libitum* for a period of 110 days.

Late adverse experience (E2): (alteration of the social bond through isolation in adulthood).

Newborn rats with 23 days of lactation were separated from their mother and kept in a cage

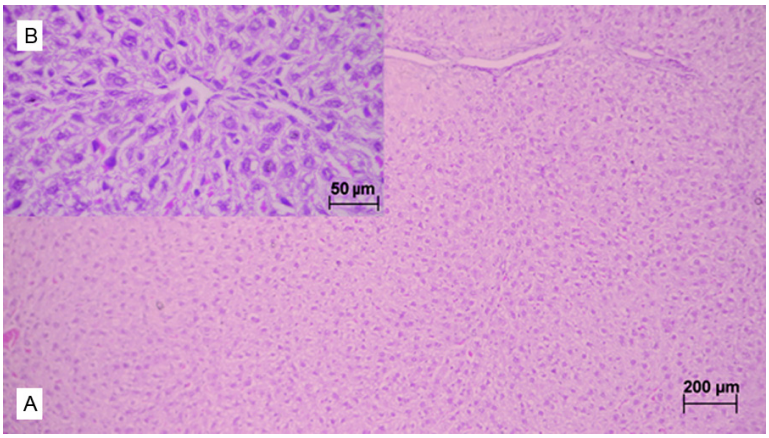

Figure 3. Liver of Sprague-Dawley rat, E2 group. A. Disorganization of the hepatic cords is observed. B. Larger hepatocytes and hydropic degeneration. H&E staining.

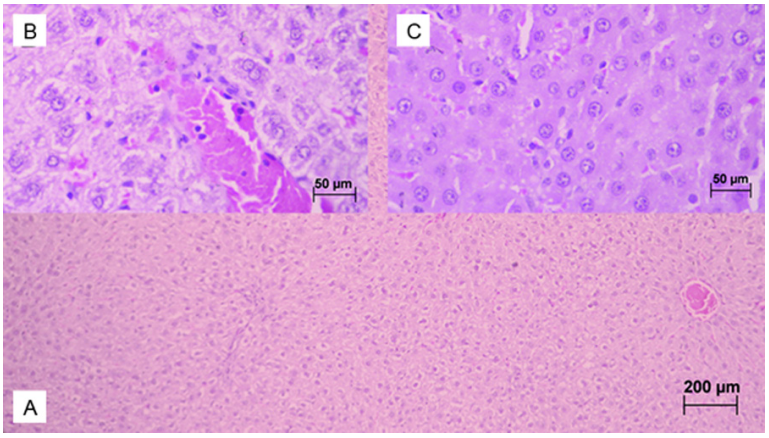

Figure 4. Liver of Sprague-Dawley rat, E3 group. A. Greater disorganization of the hepatic cords is observed. B. Focus with slight, mixed inflammation. C. Regenerative focus with hepatic cells with anisocytosis. H&E staining.

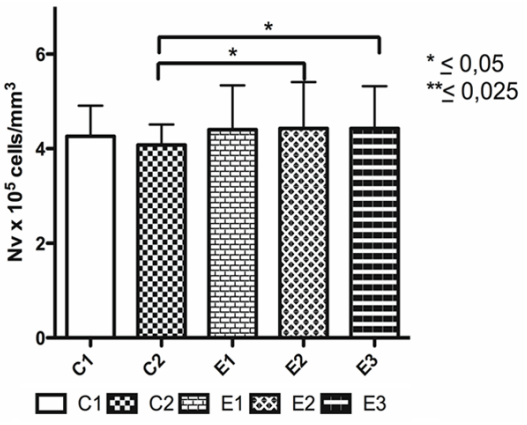

Figure 5. Number density in rat liver. Average hepatocyte number density of Sprague-Dawley female rats previously subjected to early (E1), late (E2), and early-late (E3) adverse experiences and later intermittent chronic stress in adulthood.

under conditions of social interaction, with water and food (pellets) *ad libitum* for a period of 80 days. Then, they were placed in individual cages, restraining all types of social interaction for 110 days. During this period they had access to water and food (pellets) *ad libitum.*

Early-late adverse experience (E3)

Newborn rats with 18 days of lactation were separated from their mother and kept in a cage under conditions of social interaction, with water and food (pellets) *ad libitum* for a period of 80 days. Then, they were placed in individual cages, restraining all types of social interaction for 110 days. During this period they had access to water and food (pellets) *ad libitum.*

Control group 1 (C1)

Group without early or late adverse experiences and without exposure to intermittent chronic stress (normal morphologic control of the liver).

Newborn rats with 23 days of lactation were separated from their mother and kept in a cage under conditions of social interaction, with water and food (pellets) *ad libitum* during the entire experiment (116 days).

Control group (C2)

Newborn rats with 23 days of lactation were separated from their mother and kept in a cage under conditions of social interaction, with water and food (pellets) *ad libitum* for a period of 110 days.

Measuring intake behavior

At the end of the 110-day period, for the following 6 days, the members of each group were deprived of food for 20 hours a day. Afterwards, for 2 hours (anticipatory period), in the pres-

Table 1. Statistical analysis results for the average hepatocyte Nv in Sprague-Dawley female rats subjected to early and late adverse experiences and later intermittent chronic stress

Nv	Average (cells/mm^3)	SD	Minimum (cells/mm^3)	Maximum (cells/mm^3)	SE (cells/mm^3)	CV (%)	CE (%)	*P* value
C1	4.26 x 10^5	0.65	3.85 x 10^5	5.06 x 10^5	0.29 x 10^5	15.18	6.79	0.902
C2	4.08 x 10^5	0.43	3.82 x 10^5	4.66 x 10^5	0.19 x 10^5	10.29	4.60	0.214
E1	4.40 x 10^5	0.94	3.84 x 10^5	5.67 x 10^5	0.42 x 10^5	21.30	9.53	0.243
E2	4.43 x 10^5	0.98	3.84 x 10^5	5.82 x 10^5	0.44 x 10^5	22.10	9.88	0.249
E3	4.43 x 10^5	0.89	3.85 x 10^5	5.53 x 10^5	0.40 x 10^5	20.10	8.99	0.642

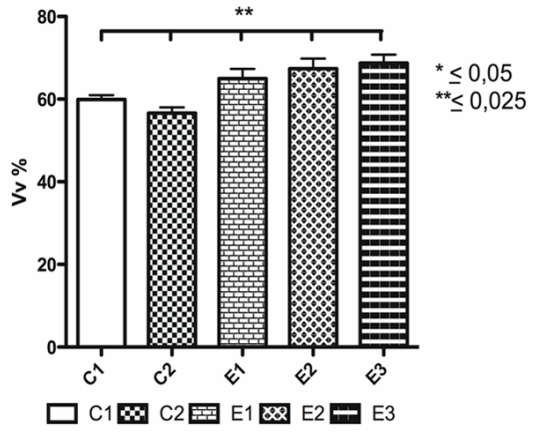

Figure 6. Volume density in rat liver. Average hepatocyte volume density of Sprague-Dawley female rats previously subjected to early (E1), late (E2), and early-late (E3) adverse experiences and later intermittent chronic stress in adulthood.

ence of a visual stimulus (red light), they were provided with a diet of 50 g of pellets and 50 g of Quaker Quadritos® (oatmeal squares) with 200 ml of water. At the end of this period, the food and water were removed to measure intake. Then, for another two hours (stress stage), the rats were given the same diet, but this time in the presence of the stress stimulus. At the end of this period, the stress stimulus was removed, and the food and water intake was quantified. The intake behavior was evaluated by determining the number of calories consumed per each gram contributed by the diet.

Diet

Pellet: 5% fiber and 20% protein (3.375 cal/g).

Quaker Quadritos®: 4.5% fat, 11% protein, and 70% carbohydrate (3.640 cal/g).

Anticipatory stage

For a period of 2 hours a day for 6 consecutive days, a visual stimulus (red light) was applied to each rat as previously described before intermittent chronic stress.

Intermittent chronic stress stage

For a period of 2 hours a day after the anticipatory stage and for 6 consecutive days, a stress stimulus (tail pinch) was applied to each rat, which consisted of placing a metallic clamp approximately 2 cm distal to the base of the tail [29].

Histologic and stereologic analysis

On postnatal day 116, the rats were sacrificed by cervical dislocation to extract the liver. For the stereologic study, we obtained five random pieces of each liver, considering the isotropic characteristics of the tissue. Subsequently, they were fixed in 10% buffered formalin for 24 hours, dehydrated, and embedded in Histosec paraffin (Merck). Once the blocks were obtained, 4 μm sections were taken on a microtome (Microm HM 325). From each block, 5 sections were made and stained with H&E. Five fields were observed for each section; in total 125 fields were observed per group [30]. The laminae were observed under an Olympus® CX31 optical microscope. The images were projected onto a flat screen monitor. The multipurpose testing system M42 was used for the stereology. The parameters measured were hepatocyte number density (Nv), volume density (Vv), and surface density (Sv).

Statistical analysis

The statistical analysis was done with the IBM SPSS Statistics 21© software, and the assumptions were verified with the one-sample Kolmogorov-Smirnov test (data normality test) and Levene's test (homoscedasticity analysis). For the analysis of the differences between groups, a one-way ANOVA and Scheffe's post hoc test were used. The *P* values were consid-

Table 2. Statistical analysis results for the average hepatocyte Vv in Sprague-Dawley female rats subjected to early and late adverse experiences and later intermittent chronic stress

Vv	Average %	SD	Minimum %	Maximum %	SE %	CV (%)	CE (%)	*P* value
C1	59.89	1.09	58.80	61.07	0.49	1.81	0.81	0.542
C2	56.61	1.38	54.74	57.79	0.62	2.44	1.09	0.572
E1	64.95	2.38	62.61	67.94	1.06	3.66	1.64	0.309
E2	67.35	2.46	64.61	70.11	1.10	3.65	1.63	0.938
E3	68.74	2.01	66.78	70.97	0.90	2.92	1.31	0.761

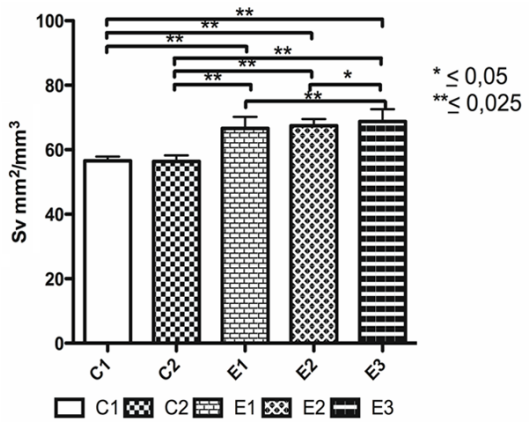

Figure 7. Surface density in rat liver. Average hepatocyte surface density of Sprague-Dawley female rats previously subjected to early (E1), late (E2), and early-late (E3) adverse experiences and later intermittent chronic stress in adulthood.

ered significant at less than 0.05 (*) and very significant at less than 0.025 (**).

Results

Histologic analysis

Control group C1 presented a preserved lobular architecture, i.e., radial hepatocyte chains from the central vein and separated by apparently normal sinusoids. The portal area exhibited the three elements of the portal triad. The hepatocytes had a polygonal shape, with central, round nuclei and eosinophilic cytoplasm (**Figure 1**).

Control group C2 also presented a preserved lobular architecture. Nevertheless, in some areas distant to this pericentrolobular area, there was disorganization of the hepatic cords. The characteristics of the hepatocytes, sinusoids, and portal triad were within normal limits. However, some foci were observed to have slight hydropic degeneration.

In experimental groups E1 and E2, the structure was maintained primarily in the area next to the central vein. On the periphery, the hepatocytes presented disorganization of the hepatic cords. Due to the presence of grouped hepatocytes, the sinusoids were more compressed and without the characteristic radial organization. Nuclear anisocytosis, larger hepatocytes, and, in some, slight hydropic degeneration were observed focally (**Figures 2** and **3**).

Experimental group E3 presented greater alteration of the lobular structure than groups E1 and E2, with some areas being preserved and others not. The presence of regeneration foci without parallel necrosis foci and relevant inflammation was observed. Inflammation was mixed, primarily lympho-plasmatic, and there was hydropic degeneration in a greater number of cells. In the regenerative foci, there was an increase in hepatocyte anisocytosis and nuclear staining, as well as an increased presence of binucleated cells (**Figure 4**).

Stereologic analysis

In terms of the average hepatocyte number density, the ANOVA revealed at least one group that differed from another (F_{calc} = 4.019; *P* = 0.003).

Scheffe's post hoc test showed significant differences in the average hepatocyte Nv, with groups E2 (4.43 x 10^5/mm^3; SD 0.98) and E3 (4.43 x 10^5/mm^3; SD 0.89) having greater values than group C2 (4.08 x 10^5/mm^3; SD 0.43; *P* = 0.030); there were no significant differences with groups C1 (4.26 x 10^5/mm^3; SD 0.65; *P* = 0.651) and E1 (4.40 x 10^5/mm^3; SD 0.94; *P* = 0.999) (**Figure 5**). **Table 1** shows the results of the intragroup statistical analysis of the average hepatocyte Nv of the study groups.

The ANOVA for the average hepatocyte volume density showed at least one group that differed from another (F_{calc} = 752.779; *P* = 0.000).

The average hepatocyte volume density was greater in group E3 (68.74%; SD 2.01) and

Table 3. Statistical analysis results for the average hepatocyte Sv in Sprague-Dawley female rats subjected to early and late adverse experiences and later intermittent chronic stress

Sv	Average (mm^2/mm^3)	SD	Minimum (mm^2/mm^3)	Maximum (mm^2/mm^3)	SE (mm^2/mm^3)	CV (%)	CE (%)	*P* value
C1	56.56	1.29	54.37	57.11	0.58	2.29	1.02	0.835
C2	56.35	1.91	53.61	57.72	0.85	3.39	1.51	0.979
E1	66.65	3.54	63.21	70.92	1.58	5.32	2.38	0.962
E2	67.44	2.07	65.04	69.27	0.93	3.07	1.37	0.232
E3	68.78	3.77	65.07	73.26	1.69	5.46	2.44	0.402

lower in group C2 (56.61%; SD 1.38). Scheffe's post hoc test showed highly significant differences ($P = 0.000$) among all the study groups (**Figure 6**). **Table 2** shows the results of the intragroup statistical analysis of the average hepatocyte Vv of the study groups.

In terms of the average hepatocyte surface density, the ANOVA revealed at least one group that differed from another ($F_{calc} = 434.149$; $P = 0.000$).

The experimental groups had a greater average Sv than the control groups ($P = 0.000$); group E3 presented the highest value (68.78 mm^2/mm^3; SD 3.77), followed by E2 (67.44 mm^2/mm^3; SD 2.07) and E1 (66.65 mm^2/mm^3; SD 3.54). There were no significant differences between E1 and E2 ($P = 0.275$). Compared to the control groups, differences were not statistically significant ($P = 0.992$). **Figure 7** presents the results of Scheffe's post hoc analysis of the average hepatocyte Sv. **Table 3** shows the results of the intragroup statistical analysis of the average hepatocyte Sv of the study groups.

Discussion

The consequences of acute and chronic stress have been shown to include an association with functional [13-15, 18] and morphologic changes in the liver, with necrosis [17] and regeneration [22] as the main structural changes. On the one hand, necrosis is linked to degenerative changes that lead to an increase in the size of the hepatocyte, presence of cytoplasmic vacuoles, rudimentary cytoplasm, and radial disorganization of the hepatic cords and sinusoids, whereas regenerative changes are associated with the presence of grouped nuclei, anisocytosis, binucleated cells, and the characteristic loss of radial organization.

It is important to consider that these morphologic changes are observed in experimental models in which different types of stressors and exposure time are used. However, in the stress history of the subject, the stage of life at which the stress was suffered also becomes relevant [31]. Thus, in our study, the observed morphologic changes in the liver of rats in the experimental groups are not only similar to the results described by previous authors but also increased in the most vulnerable group, i.e., the group exposed to an early adverse experience by alteration of the mother-infant social bond and of the late social bond by isolation in adulthood.

Degeneration and necrosis in the hepatic parenchyma, together with continuous hepatocyte proliferation, confirm the regenerative power of the liver [21, 22]. This process is associated with signaling pathways that involve growth factors and cytokines, particularly TNF-α and IL-6, secreted by stellate macrophages, which appear to be important mediators in the initial regeneration mechanisms [32]. In our study, early weaning and social isolation potentiate stress from the hormones in the HPA axis, which can increase plasma corticosterone levels [9, 33], which may be of benefit to the production of TNF-α and IL-6 by stellate macrophages. Thus, as previously indicated, the regenerative characteristics of the liver, along with the applied model, could in part explain the correlation observed between the histologic changes produced and the stereologic results found.

Sharma [21] and Agrawal and Gupta [22] observed an increase in hepatocyte Nv and Vv as the frequency of exposure to heat stress increased. In our study, despite differences in the stressor, higher values in all the stereologic parameters analyzed were found in the experimental groups compared to control group C1, with very significant differences ($P < 0.025$) in hepatic Vv and Sv for groups E2 and E3. These results might reflect hepatocyte hypertrophy

associated, on the one hand, with the degenerative process with an increase in size due to hydropic degeneration and, on the other, with the hepatic regeneration that leads to an increase in the size of the hepatocyte when it is binucleated. Regeneration is also reflected in the increased hepatocyte Nv values, more evidently in group E3. Radial disorganization of the hepatic cords, compressed sinusoids, anisocytosis, and grouped hepatocytes might favor a greater number of cells per mm^3.

The stereologic parameters were lower in group C2 than in group C1; however, the differences were significant only for Vv ($P = 0.000$). These results seem to indicate less regenerative activity, which might be explained by the liver beginning the regeneration stage hours after the functional need or days after the removal of part of the organ [34]. In our study, group C2 was exposed to intermittent chronic stress and then sacrificed immediately after the intervention.

We can conclude that exposure to chronic stress can affect the hepatic morphology in Sprague-Dawley rats, which is consistent with Nadal and Armario [35], who reported that the effects of stress are related to the nature, intensity, and duration of the stress stimulus. Nevertheless, when individuals have been subjected to previous adverse experiences, such as early weaning or social isolation in adulthood, the morphologic changes in the liver are more evident.

Disclosure of conflict of interest

None.

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