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Original Article

Protective effects of insulin treatment in the morphological alterations and oxidative damage to DNA in the liver of young rats subjected to skin scald burn injury

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Abstract: Background: Burn injury (BI) represents a major epidemiologic problem worldwide, mostly in children. BIs greater than 40% of the total body surface, are considered severe, and entail a hepatic hypermetabolic response, which is associated with proteins depletions and prolonged hypermetabolism. Objective: This study aims to evaluated the effects of short- and long-term insulin treatment on liver morphology and the use of a biomarker related to oxidative damage to DNA (8-OHdG) to better understand the anabolic action of this hormone in the liver. Methods: Wistar rats aged 21 d were distributed into four groups: control (C), control with insulin (C+I), scald burn injury (SBI), and SBI with insulin (SBI+I). The SBI groups were subjected to a burn 45% total body surface area. The C+I and SBI+I groups received insulin (5 UI/Kg/d) for 4- or 14 d. The livers were analyzed for morphometric, histopathological, and immunohistochemical for 8-OHdG. Results: The main results showed that, in a short time, insulin increases the density of binucleated hepatocytes as an organ response to burn injury. In the long term, insulin increased the area of hepatocytes in the SBI+I group in relation to SBI, highlighting the similar values between the SBI+I and the control groups. Regarding sinusoidal cells, insulin was able to modulate this liver proliferative reaction. Insulin reduces 8-OHdG immunoexpression in short and long-term post-burn moments. Conclusion: The insulin modulation of 8-OHdG makes us infer that a study about the control of 8-OHdG as a potential biomarker in patients could be an efficient precursor of the level of oxidative stress associated with hepatic dysfunction associated to extensive burn injury.

Keywords: Thermal injury, liver, 8-OHdG, hypermetabolism, morphology

Introduction

Burn injury (BI) represents a major epidemiological problem worldwide, mostly in children aged zero and five years old [1, 2]. Due to scalding, BIs are common among children, usually occurring in a domestic environment [3]. When the BIs are greater than 40% of total body surface, they are considered severe and entail a hepatic hypermetabolic response, which is associated with proteins depletion [4]. Such outcome that can persist up to 36 months in severely burned children, demonstrating prolonged hypermetabolism [5]. Burn size determinates the hypermetabolic response [6] and the accelerated protein breakdown is not rectified by its synthesis, resulting in net protein losses [7].

The increase of energetic expenditure in the body due to the high loss of body heat and the hepatic hypermetabolic condition leads to a raise in metabolic processes like gluconeogenesis, fatty acid synthesis and the Cori cycle [8]. To maintain liver functioning throughout the hypermetabolic stress caused by burn injury, the Cori cycle converts lactate into pyruvate producing glucose by anaerobic metabolism. Stress generates hyperglycemia due to glycogenolysis, gluconeogenesis, lipolysis, and protein catabolism [9]. This response to hypermetabolic stress occurs because of the inflammatory

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process encompassing hormones, cytokines, and acute phase proteins that are produced by the liver [10]. The liver is unable to metabolize all the accumulating substances and leading to hepatomegaly, which is associated with the presence of hyperlipidemia and hyperglycemia with insulin resistance. Such condition worsens the hypermetabolic and inflammatory state and, if hypermetabolism cannot be decreased, it may lead to multiple organ failure and death [11].

Hepatic dysfunction can lead to cell damage with leaked hepatic enzymes in the bloodstream like aminotransferase and alanine aminotransferase, which are main indicators of hepatocyte injury [12]. The liver damage has been associated with hepatocyte death boostered by necrosis and apoptosis. To offset the imbalance, an increase of hepatic cells proliferation has been found, suggesting that the liver tries to maintain the homeostasis [13]. Moreover, there is a fatty infiltration in severe burn injuries due to the free fatty acids and triglycerides increase (due to carrier proteins decrease), which are associated with elevation of bacterial translocation, hepatic insufficiency, and endotoxemia [14]. Liver fatty acids absorption accelerates lipolysis resulting in a gain in the rate of hepatic absorption required for oxidation, thus, resulting in hepatic steatosis [12].

Due to hypoglycemia and insulin resistance related to hypermetabolism and inflammatory response [15] the insulin treatment after thermal injury has shown positive results that can significantly contribute to mortality and morbidity reduction [16], and a reduction in resting energy expenditure in severely burned children [17]. Insulin is an efficient anti-inflammatory (for decreasing TNF-alpha levels) and antioxidant, and it can be used as supporting treatment for multiple organ dysfunction after trauma [18].

In addition to all consequences of hypermetabolic and inflammatory state in extensive burns, disturbances in the cellular redox occur [19]. Changes on cellular redox play a significant role, affecting the cell response to oxidative stress [20], which is identified when there is an overproduction of reactive oxygen species (EROs), insufficient EROs detox by antioxidants or the combination of both, representing an imbalance between pro-oxidant and antioxidant cell potential [21]. Such reactive species work like essential metabolites that compromise biological processes and can result in inflammatory diseases, cancer, diabetes mellitus and other disturbances [22-24].

The guanine nucleotide has the lowest reduction potential, and it is preferably oxidized. One of the resulting products of such process is the formation of 8-OHG, which consequently undergoes another reaction and changes into 8-hydroxy-deoxyguanosine (8-OHdG) [24-26]. Beyond the damage on the deoxyribonucleic acid (DNA) structure by guanine loss, the 8-OdG transformation can entail secondary DNA adducts, which have tumorigenesis induction as a consequence [21]. The 8-OHdG species is used like oxidative DNA damage biomarkers because it is not metabolized or degraded, being extracellularly discharged and eliminated by urine [26]. Moreover, when patients with chronic C hepatitis have the same species accumulation in Kupffer cells and hepatic inflammatory cells on periportal area (zone 1) as well as an accumulation in hepatocytes nucleus at the same region, that indicates a high tissue inflammation degree [27].

Therapeutic approaches to extensive BIs have aimed to reverse hypermetabolic response based on anabolic agents such as growth hormones, insulin, Insulin-like growth factor 1 (IGF-1), testosterone and oxandrolone [28], and anticatabolic agents such as propranolol to reduce thermogenesis, cardiac work, resting energy expenditure, and peripherical lipolysis [29]. Our previous studies of with insulin treatment in burn injury murine model found improved in elastic-collagen rearrangement and reepithelization in skin wound healing [30], as well as myogenesis modulation as a result of muscle atrophy post-burn [31]. Considering the hypermetabolic consequences of severe BI in the liver and employing insulin as a therapeutic approach, we evaluated the effects of short-and long-term insulin treatment on liver morphology and the use of a biomarker related to oxidative damage to the DNA (8-OHdG) to better understand the anabolic action of this hormone in the liver.

Material and methods

Study design and experimental groups

Forty male prepubescent Wistar rats with 21 days of age were distributed in four groups:

Control (C), Control with insulin treatment (C+I), scald burn injury (SBI), and scald burn injury with insulin treatment (SBI+I). Each group was further distributed into different euthanasia periods (4 and 14 days, n = 5). The animals were obtained from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) at the Universidade Federal de São Paulo. The animals were maintained in individual cages at temperature room (22°C) and a 12-hour light-dark cycle. Water and food were offered *ad libitum,* and all procedures were approved by the Research Ethics Committee from Universidade Federal de São Paulo (protocol no. 2520120319).

Scald burn protocol and insulin treatment

Animals, including control groups, were anesthetized with an intraperitoneal injection of Ketamine (50 mg/mL) and Xylazine (10 mg/mL), and their dorsal and ventral hair were shaved. SBI and SBI+I groups were subjected to nonlethal scald burn injury in 45% of total body surface area (30% in the dorsal and 15% in the ventral area, distant from joints), in 87°C water [32, 33]. The C and C+I group were subjected to sham scald burn injury. All groups received analgesic morphine (10 mg/Kg), which was administered subcutaneously and immediately before sham or scald injury. Morphine was administered again 24 hours later, and the animals were daily monitored for signs of discomfort or pain [34].

Immediately after scald burn injury or sham procedure, the animals from C+I and SBI+I groups were treated with insulin via subcutaneous administration (5 UI/Kg/d) of slow-acting insulin glargine Lantus® (Sanofi-Aventis Farmaceutica Ltda, São Paulo, Brazil), whereas C and SBI groups received saline solution. The insulin dosage used in this study was selected based on previous research in which insulin treatment was administered in euglycemic murine models [30, 35]. To maintain euglycemia, the insulin-treated rats received 5% sucrose in drinking water [36]. Glucose homeostasis in all animals was evaluated via blood glucose level using Accu-Chek® active glucometer (Roche Diagnóstica Brasil Ltda) through a cut at the tip of the tail of each animal. Four and 14 days following the SBI or sham procedure, all animals were euthanized with a lethal IP injection of Ketamine (150 mg/kg) and Xilazyne (30 mg/kg).

Morphological analysis of liver cells

The animals' livers of C, C+I, SBI and SBI+I groups were dissected and the right lobe fragments (1-2 cm) were fixed on Phosphate Buffered Saline (PBS) formaldehyde solution. The specimens were stored in 70% alcohol and, posteriorly, routinely embedded in paraffin blocks and cut in 4 µm thickness. The slides with liver specimens were stained with Hematoxylin and eosin (H&E), a routine technique that uses hematoxylin to stain cell nuclei in dark purple and eosin to stain the extracellular matrix and cell cytoplasm pink. In addition, Sirius-Red stain was used together with polarized light to differentiate type I (red) and III (green) collagen. Photomicrographs were made by computerized image equipment (AxioVision 4.5, Carl Zeiss, Oberkochen, Germany) attached to a binocular microscope (Axioscope Observer D1 Carl Zeiss).

Hepatocyte area and cell density were determined by morphoquantitative liver cells analysis. The hepatocyte area (μm^2) was determined based on the measurement of 50 cells, stained with H&E, and randomly chosen per animal of each experimental group. Cell density (number of cells/mm^2) was determined based on mononucleated hepatocytes, binucleated hepatocytes and sinusoidal cells [33]. For this purpose, five cuts that were chosen randomly from ten sections stained with H&E were used. A total of 10 photomicrographs were assessed per animal to determine liver cells density. Computerized imaging equipment (AxioVision 4.5, Carl Zeiss, Oberkochen, Germany) attached to a binocular microscope (Axioscope Observer D1 Carl Zeiss) with a 40x objective was used.

8-OHdG immunohistochemical analysis

The liver sections (4 µm) were removed and pre-treated with citric acid buffer (pH = 6) to perform antigen recovery. The sections were pre-incubated for 5 min in 0.3% hydrogen peroxide in PBS solution to inactivate the endogenous peroxidase. The material was blocked with 5% normal goat serum in PBS solution for 10 min and then incubated over-

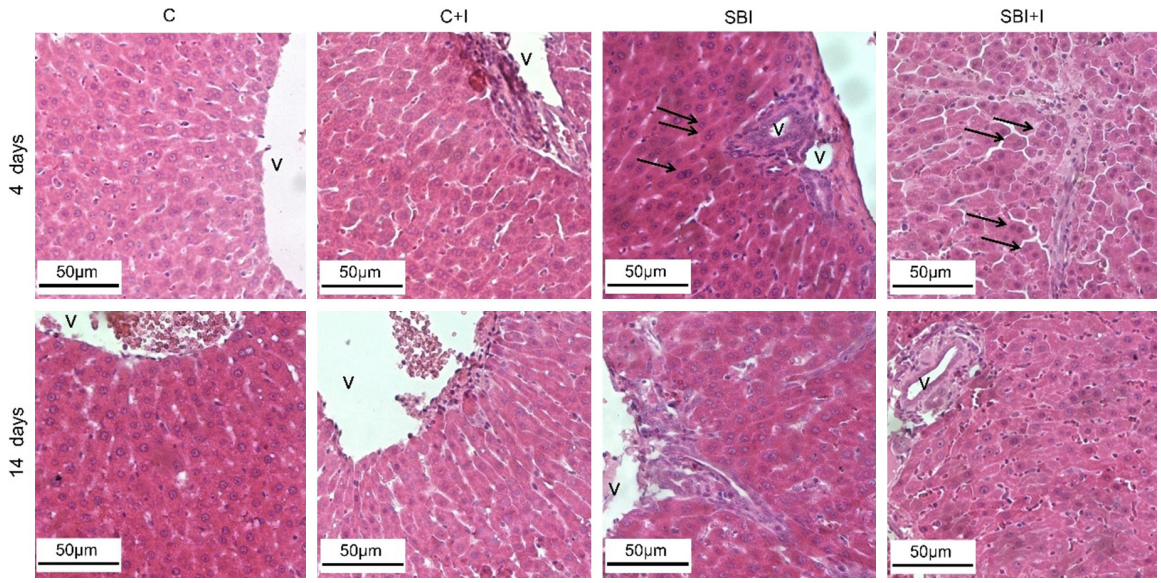

Figure 1. Histopathological liver evaluation of C; C+I; SBI and SBI+I groups with 4 or 14 days after burn injury stained with hematoxylin and eosin. Note the thickened wall vessels (v) in SBI and SBI+I and numerous binucleate hepatocytes, more expressive in the 4 days groups. Sample size: n = 10 per group. Hematoxylin & eosin. Bar = 50 µm.

night at 4°C in a refrigerator with mouse monoclonal anti-8-OHdG (dilution of 1:200, Santa Cruz Biotechnology-66,036). After PBS washing, the tissue was incubated with biotin-conjugated anti-rabbit secondary antibody IgG, followed by streptavidin peroxidase, and stained with DAB chromogen of the Starr trek Universal HRP Detection kit (Biocare Medical, California, USA), contrasted with Harris' hematoxylin.

8-OHdG immunostaining was evaluated by a semi-quantitative score as follows: 0 for 0 < 5%; 1 for 5-25%; 2 for 25-50%; 3 for 50-75%; and 4 for 75-100% immunostaining area [37]. The analysis was performed independently by two observers. All photomicrographs were obtained with an Axio Observer. D1 microscope (Zeiss, Ny, USA) coupled to a computerized imaging system (Axio Vision 4.8. Zeiss software).

Statistical analysis

Statistical analysis for liver cells density and 8-OHdG score were evaluated by analysis of variance (ANOVA) with two fixed factors (group and treatment) and followed with a Tukey's test for multiple comparisons. To statistical significance was considered $P < 0.05$.

Results

Effects of insulin on liver cells morphology

Histopathological liver cuts stained by H&E (**Figure 1**) revealed (SBI and SBI+I) groups with thickened wall vessels in both experimental periods, but more expressive in the 4 days groups. Furthermore, that region showed numerous binucleate hepatocytes confirming the morphoquantitative findings.

Hepatocytes area was significantly higher in the SBI+I group that was investigated 14 days post BI when compared with SBI, $P < 0.05$ (**Figure 2A**). In this period, average area values of SBI+I group were similar to control group. There was no difference in comparison with other groups.

Regarding mononucleated hepatocyte cell density (cells number/mm^2) represented in **Figure 2B**, no differences between groups were observed. Binucleated hepatocyte cell density (**Figure 2C**) analyzed 4 days post injury showed higher density in SBI and SBI+I groups compared with C and C+I ($P < 0.05$) and SBI showed higher binucleated cell density compared with C+I ($P < 0.05$). No differences were observed 14 days after injury. Sinusoidal cells (**Figure 2D**) showed higher density in SBI

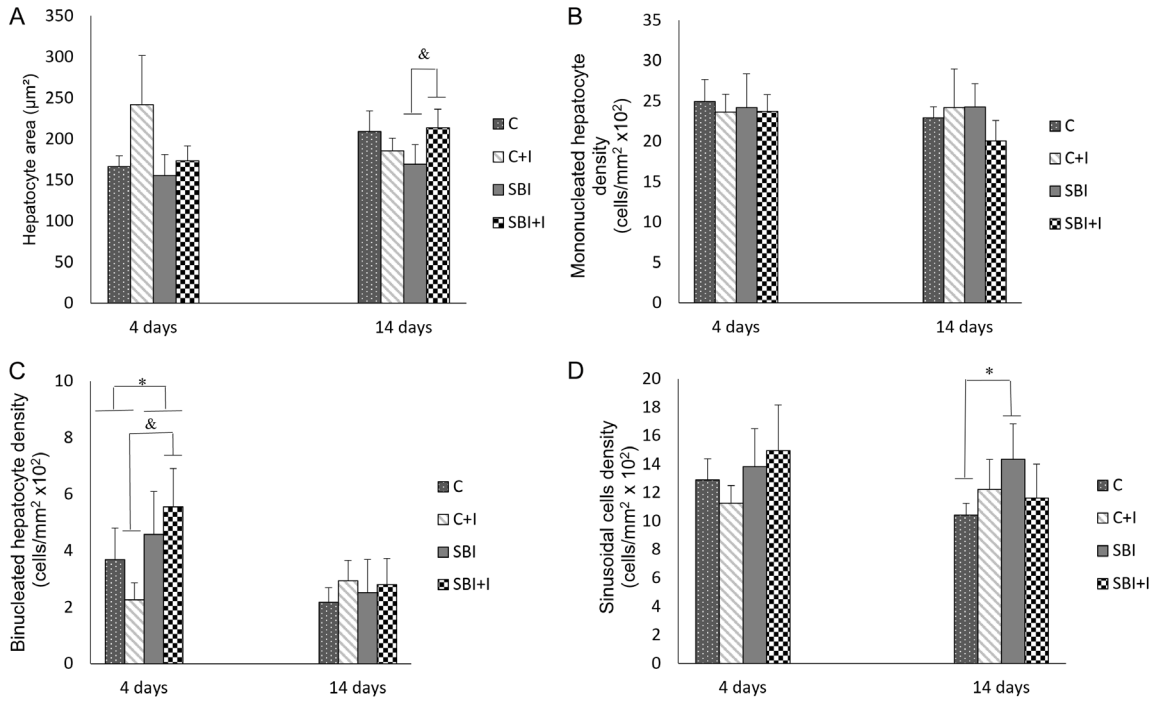

Figure 2. Hepatocyte area (µm^2) (A), mononucleated hepatocyte density (B), binucleate hepatocytes density (C) and sinusoidal cells density (D) of control group (C) control group + insulin treatment (C+I), Scald burn injury (SBI); and Scald burn injury + insulin treatment (SBI+I), evaluated within 4 and 14 days. SBI+I group had higher hepatocyte area than SBI, &P < 0.05. No difference was observed in mononucleated hepatocyte density groups. Binucleated hepatocytes density had significantly increased within 4 days in SBI and SBI+I group compared to controls (*P < 0.05) and SBI+I animals presented higher density than C+I, 4 days post injury (&P < 0.05). Sinusoidal cells increase density were higher in SBI when compared with C, *P < 0.05, 14 days post injury. Sample size: n = 10 per group.

compared with C 14 days post injury, P < 0.05, and no differences were observed 4 days after injury.

The histopathological connective tissue analysis (**Figure 3**) revealed well-delimited vessels walls composed of type I (red and orange) and type III (green) collagen fibers. The thickened walls in SBI and SBI+I vessels observed with H&E staining were confirmed by Sirius-red technique under polarized light. However, it is important to highlight collagen fibers irradiation (arrows) starting from vessel walls to the hepatic parenchyma of scalded burn groups and with higher intensity in SBI without insulin treatment, 14 days post burn.

8-OHdG immunohistochemistry

8-OHdG immunohistochemistry related to oxidative stress with DNA damage (**Figure 4**) was identified predominately in SBI group with numerous marked hepatocyte nuclei 4- or 14-days post injury. SBI and SBI+I groups had higher 8-OHdG immunohistochemical score values than C and C+I (P < 0.05), in both periods. The insulin modulates 8-OHdG immunoexpression since the SBI+I group showed a lower score than SBI in both periods (P < 0.05).

Discussion

Thermal injuries over 40% of the total body surface are considered extensive. Such condition, in addition to local responses, lead to systemic repercussions that cause a state of hepatic hypermetabolism, protein catabolism, insulin resistance and growth delay and, therefore, changes in liver morphology. Thus, based on adaptive changes in the liver in response to extensive thermal injuries, this study aimed to assess whether the effects of insulin treatment in the short- and long-term (4 and 14 days) after extensive thermal injury would be able to alter or delay adaptive morphological changes in the liver.

In the analysis of long-term outcomes, insulin was able to increase the area of hepatocytes in SBI+I compared to the SBI group. Such

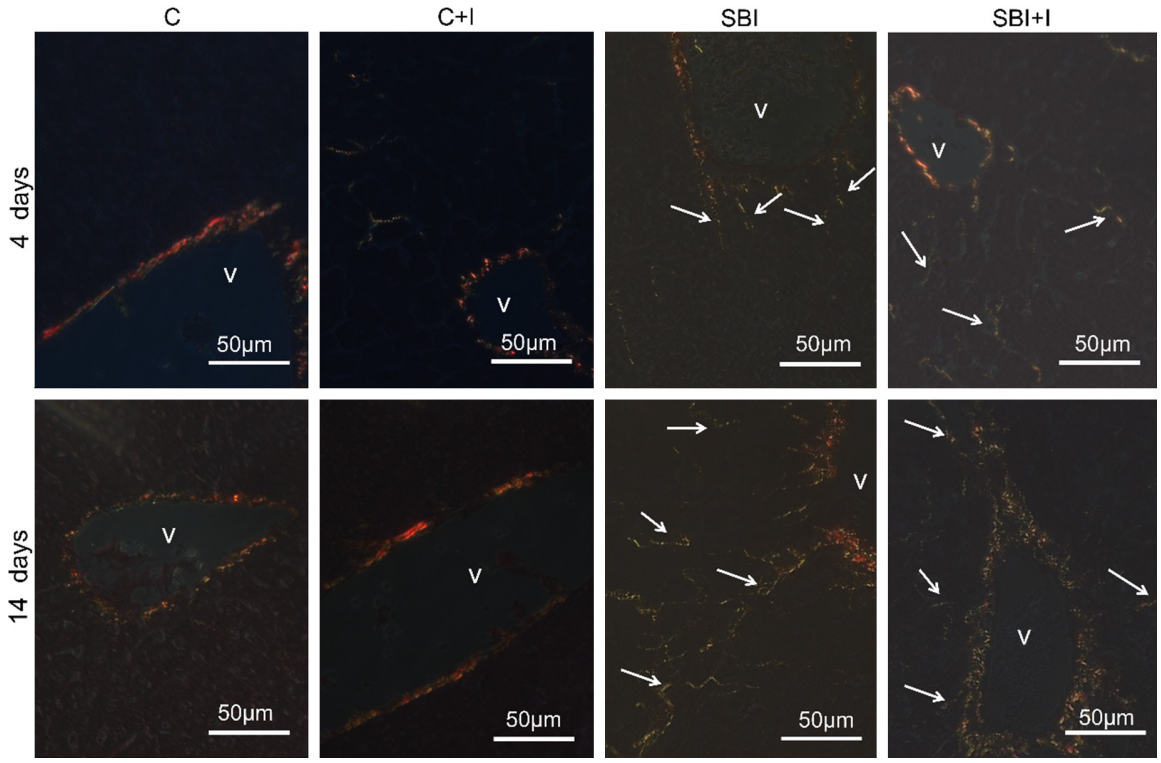

Figure 3. Histopathological liver connective tissue analysis of C; C+I; SBI and SBI+I group with 4 and 14 days after burn injury. "V" represents a well-delimited vessels walls with collagen fiber type I (red and orange) and type III (green). Arrows demonstrate collagen fibers irradiations from the vessel wall to hepatic parenchyma SBI groups. Sample size: n = 10 per group. Sirius-red under polarized light, bar = 50 μm.

increase corresponds to an attempt of tissue regeneration in the lesion. This indicates that insulin was able to increase the regeneration stimulus of the injured tissue, since the SBI+I group had a larger area of hepatocytes than the SBI group.

Previous liver study with extensive burn injury, have already demonstrated that scalded burn injury rats showed an increase of the hepatocyte area compared to control group in 14 days [33], therefore corroborating our study, and specially our outcomes on the insulin boosting effect. Insulin brought the values of the SBI+I group closer to the reference control value, which shows that the dose, frequency and period in which it was administered were unable to increase the average hepatocyte area to values similar to that found in the control. If it occurs, it must be analyzed with caution, as for the tissue regeneration, because burn injuries are able to induce hepatomegaly in individuals who suffered extensive thermal burn injury [8, 9]. Such disturbance could reduce sinusoidal circulation causing hypoxia and tissue necrosis [38].

Although no significant results were observed in short- and long-term analyses regarding mononucleated hepatocytes density, a study [33] suggests a decrease in these cells in the SBI groups on experimental days 1 and 14 and describes it as a relationship with the degeneration process found in the pathological processes of the research. Despite insulin treatment not modifying the mononucleated cells density, it was established that this hormone directly amplifies the mitogenic signals of hepatocyte growth factor (HGF) and intensifies the liver regeneration stimulus [39]. After severe trauma HGF was able to reduce hepatic inflammatory response by decreasing the pro-inflammatory cytokines messenger ribonucleic acid (mRNA) transcription and the expression of caspases 3 and 9, thereby increasing the proliferation of hepatocytes and improving liver morphology [39].

Our results showed a greater increase in binucleate hepatocytes density for SBI and SBI+I groups when compared to C and C+I treatment in the 4-day analysis. Such outcome corroborates with a study that evidenced a prolifera-

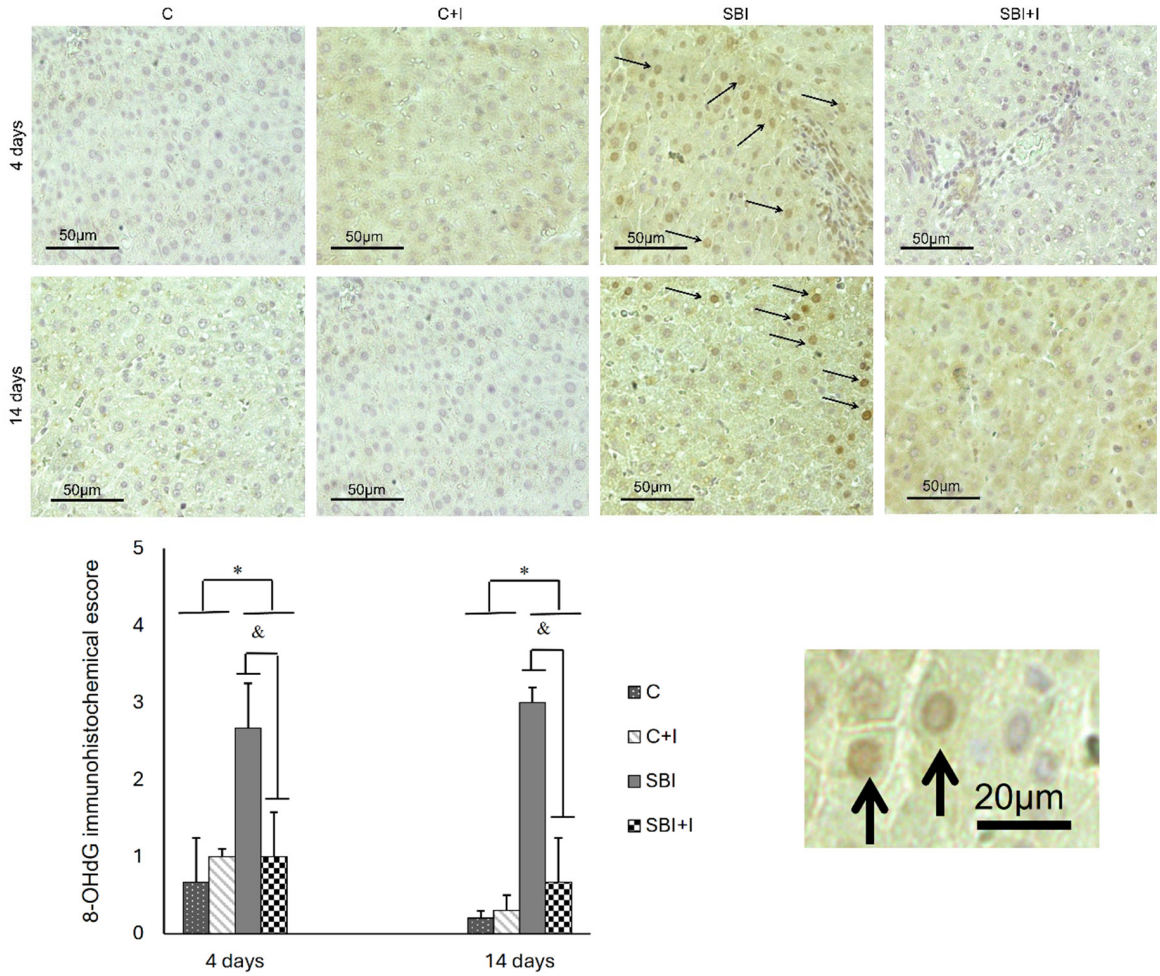

Figure 4. Rats liver 8-OHdG immunohistochemical of C; C+I; SBI and SBI+I group within 4 and 14 days after burn injury. Photomicrographs show nuclear 8-OHdG immunostaining (arrows) in SBI groups. 8-OHdG Immunohistochemical. Bar = 50 μm. The graph represents the 8-OHdG immunohistochemical score, SBI and SBI+I pretended higher score than C and C+I (*P < 0.05) in both periods investigated. SBI+I presented a lower score than SBI in both periods (&P < 0.05). To the right of graph, the image shows 8-OHdG immunoreactive nuclei considered for the score. Sample size: n = 10 per group. Bar = 50 μm.

tion of these cells in the burn group compared to controls groups in seven experimental days [40]. Also, the authors describe a peak of cells due to the regenerative growth of the organ in response to the injury, tending to homeostasis, i.e., a compensatory proliferation related to necrosis and apoptosis.

Regarding sinusoidal cells, the SBI group showed higher density of these cells in the long term when compared to the control group. The SBI+I did not show a difference between the groups, as the same of control groups, showing the capacity of insulin to mitigate the sinusoidal cells proliferation in this experimental model. The sinusoidal cells increase is related to hepatic injury and inflammatory response beyond a hepatocyte growth factor source that prevents the activation of stellate cells which change the hepatic profile resulting in fibrosis [41].

By analyzing connective tissue, hepatic morphology revealed thicker vessel walls of groups that suffered scald burn injury, regardless of treatment or experimental period. An experimental study identified that after extensive burn injury in pigs, hepatic arterial blood flow decreases in the first four hours [42]. Based on this information, a portal hypertension is observed in these data, which has, because of a selective vasoconstrictive impact, evidence

of injury due to pronounced ischemia/reperfusion.

The 8-OHdG immunohistochemical score modulates this immunoexpression, since the SBI+I in both investigated periods showed a lower score compared to SBI. The EROS increase triggers host's defense mechanisms, which include the increase in the end products of advanced glycation and this accumulation cause the thickening of vessels [43]. Although we identified thicker vessel walls in the SBI and SBI+I groups, the presence of immunohistochemical staining for the 8-OHdG antibody can be only observed in a clear and effective way in the SBI groups, stressing the role of insulin in decreasing EROS related to DNA damage.

In conclusion, the main results showed that insulin increases in the density of binucleated hepatocytes in the short term, as a liver response to burn injury. In the long term, insulin was able to increase the area of hepatocytes in the SBI+I group in relation to SBI. Notably, the area of hepatocytes in the SBI+I group was very similar to the reference value of control groups. Regarding sinusoidal cells, insulin was able to modulate the proliferative reaction of the liver. In histopathological connective analysis, insulin therapy in both experimental periods decreased the fibrosis process, as evidenced by the reduction of collagen irradiations in the liver parenchyma of the scalded burn group compared to untreated animals. Finally, insulin reduces 8-OHdG immunoexpression 4 and 14 days after burn. Studying 8-OHdG outcomes to cellular damage and the insulin modulation of its effect makes us infer that a study about the control of 8-OHdG as a potential biomarker in patients could be a good precursor of the level of oxidative stress associated with hepatic dysfunction associated with extensive burn injury.

Disclosure of conflict of interest

None.

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