SOUTH AMERICAN JOURNAL
of Basic Education, Technical and Technological

SAJEBTT, Rio Branco, UFAC
v. 7 n. 2 (2020): Edição mai/ago, p. 280-312
ISSN: 2446-4821

# MELATONIN MODULATES ANTIOXIDANT RESPONSE AND PROTECTS HEPATOCYTES IN RATS WITH SEVERE ACUTE LIVER FAILURE

# A MELATONINA MODULA A RESPOSTA ANTIOXIDANTE E PROTEGE OS HEPATÓCITOS DE RATOS COM INSUFICIÊNCIA HEPÁTICA AGUDA GRAVE

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**ABSTRACT**

Severe acute liver failure is a rare clinical condition that affects previously normal livers and is rapid and fulminant. This study aimed to investigate the effects of melatonin on hepatocyte degeneration and loss of function. An experimental study was conducted using thioacetamide to induce liver failure in Wistar rats. After total protein assay, cell membrane damage was assessed, followed by carbonyl groups assay. The concentration of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase, the level of ammonia, and the activity of superoxide dismutase and catalase were assayed by spectrophotometry. Inorganic elements were identified by particle-induced X-ray emission, the Keap1-Nrf2 pathway was determined by immunohistochemistry, and the structure of liver parenchyma was assessed by histology and by electron microscopy. Melatonin reduces lipid peroxidation and protein carbonylation, maintains liver integrity, modulates antioxidant response and ammonia levels, and preserves the architecture of the parenchyma and the structure of hepatocyte organelles.
**Keywords**: Acetamide-N-(2-(5-methoxy-1H-indol-3-yl)ethyl). Antioxidants. Oxidative stress. Free radicals.

**RESUMO**

A insuficiência hepática aguda grave é uma condição clínica rara que afeta fígados previamente normais e é rápida e fulminante. Este estudo teve como objetivo investigar os efeitos da melatonina na degeneração e perda de função dos hepatócitos. Um estudo experimental foi conduzido usando tioacetamida para induzir a insuficiência hepática em ratos Wistar. Após o ensaio de proteína total, o dano à membrana celular foi avaliado, seguido por ensaio de grupos carbonil. A concentração de aspartato aminotransferase, alanina aminotransferase e fosfatase alcalina, o nível de amônia e a atividade da superóxido dismutase e catalase foram avaliados por espectrofotometria. Os elementos inorgânicos foram identificados por emissão de raios-X induzida por partículas, a via Keap1-Nrf2 foi determinada por imunohistoquímica e a estrutura do parênquima hepático foi avaliada por histologia e microscopia eletrônica. A melatonina reduz a peroxidação lipídica e a carbonilação de proteínas, mantém a integridade do fígado, modula a resposta antioxidante e os níveis de amônia e preserva a arquitetura do parênquima e a estrutura das organelas dos hepatócitos.
**Palavras-chave:** Acetamida-N- (2- (5-metoxi-1H-indol-3-il) etil). Antioxidantes. Estresse oxidativo. Radicais livres.

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SAJEBTT, Rio Branco, UFAC
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## 1. INTRODUCTION

Severe acute liver failure (SALF) is a potentially fatal pathological state that manifests as a rare syndrome with multiple etiologies. Hepatocytes become damaged in previously normal organs, generally followed by necrosis, encephalopathies, and coma [1,2]. Mortality is high, 85% on average, and liver transplantation is considered the most effective intervention [3,4].

We found several studies in the literature that emphasize the importance of investigating the mechanisms involved in hepatocyte toxicity and their degeneration and loss of function, since their central role in metabolism of drugs and xenobiotics makes them vulnerable [5,6]. However, ethical considerations preclude conducting such studies with human beings and so it is important to develop animal models that can be used [7].

Using thioacetamide (TAA) to induce SALF is a widely-used method, because of its recognized hepatoxicity caused by production of reactive oxygen species (ROS) and free radicals (FR) and the resultant lipid peroxidation (LPO) [8]. Elevated levels of ROS and FR induce the LPO process, which is a chain reaction in which lipids that contain unsaturated bonds are attacked. This leads to structural damage because of loss of fluidity and compromised membrane integrity [9].

In living organisms, levels of FR and other ROS are controlled by a complex system of antioxidant defenses, which reduce oxidative damage and its harmful effects on biomolecules. Antioxidants have the capacity to make FR unavailable for production of reactive oxygen species (ROS), preventing formation of free radicals in chain reactions involving iron and copper [10, 11].

Antioxidants cause the cell to transfer radicals from more sensitive regions to intracellular compartments where there is less compromise if oxidative damage does take place. More specifically, hydrophobic oxidative equivalents in aqueous phases are removed via the membrane to the cytosol which, if this did not happen, could otherwise be transferred from lipoproteins to the aqueous phase of the plasma [12,13].

The role played by enzymes in cellular mechanisms is essential for control of oxygen species, which takes place in compartments for more effective neutralization. The most important systems involve the enzymes superoxide dismutase (SOD) and catalase (CAT) which act in a coordinated manner on lipid and hydrogen peroxides ($H_2O_2$). Dismutase superoxide acts on the superoxide anion, giving rise to $H_2O_2$, which is then transformed into oxygen and water by CAT [14,15].

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Some antioxidants that can be obtained from food have a structure including aromatic rings that are capable of interacting with unpaired electrons. One example is melatonin (Mel), also named N-acetyl-5-methoxytryptamine, which is found in certain foods, helping to make up a certain proportion of daily deficiencies of the hormone [16,17]. However, Mel is also biosynthesized in the pineal gland by conversion of serotonin into N-acetylserotonin, during periods of darkness or low intensity light [18,19].

The importance of understanding the effects of Mel in different organisms lies in comprehending the relationship between its concentration and its influence on prevention and reversal of cellular damage caused by oxidative stress [20]. The objective of our study is therefore to evaluate the effects of Mel in Wistar rats subjected to an experimental model of SALF induced by TAA.

## 2. MATERIALS AND METHODS

### 2.1. Bioethical considerations

Procedures were performed in the Genetic Toxicology Laboratory at the Universidade Luterana do Brasil (ULBRA) in Canoas, Rio Grande do Sul (RS, Brazil) and at the Hospital de Clínicas de Porto Alegre, Porto Alegre (RS, Brazil), after approval by the ULBRA Animal Research Ethics Committee, granted in decision number 2018/451, of 15 May, 2018.

The study complies with the ethical and legal principles defined in Law 11.794/2008 and decree 6.899/2009 and with the guidelines published by Brazil's National Council for Control of Animal Experimentation [21] and is in compliance with state animal protection legislation and local procedures for care and use of animals.

### 2.2. Experimental procedures

Twenty-eight male Wistar rats (mean weigh 300 grams) were used in this study. During the experiment, the animals were kept in cages (47 cm x 34 cm x 18 cm) lined with wood shavings, with a 12-hour light/dark cycle and a temperature range of 18° to 22ºC. Water and chow were provided ad libitum. The animals were randomized into the following four groups (n=7): 1) control (CO): four 1 mL intraperitoneal doses of the vehicle solution (0.9% NaCl) were administered; 2) control plus melatonin (CO+Mel): two 1 mL intraperitoneal doses of the vehicle solution and two doses of Mel (20 mg/Kg) were administered; 3) thioacetamide (TAA): two doses of TAA and a further two sequential doses of the vehicle solution were administered;

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and 4) TAA plus melatonin (TAA+Mel): two doses of TAA and two sequential doses of Mel were administered.

Induction of SALF was achieved by administration of two 400 mg/kg/animal doses of TAA diluted in saline and given intraperitoneally (i.p.) with an 8h interval, following the model proposed by Schemitt et al. [22], as illustrated in Figure 1.

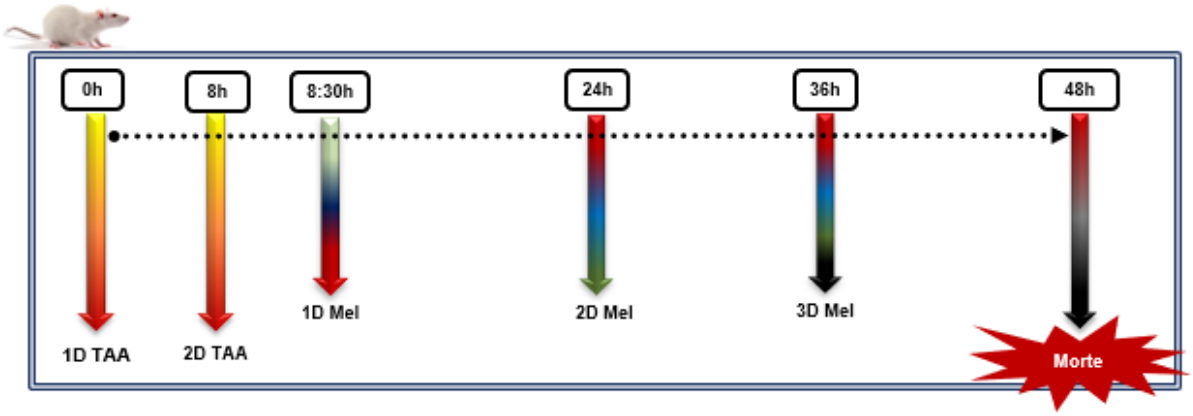


**Figure 1**. Diagram illustrating induction of SALF by administration of TAA via i.p. (400 mg/kg/animal). 1D, first dose; 2D, second dose; TAA, thioacetamide; Mel, melatonin.

The Mel-treated groups received intraperitoneal Mel (Sigma Chemical®, St. Louis, MO, United States) prepared at the time of administration, protected from light, at a dosage of 20 mg/kg per animal. Mel was administered via i.p. with a vehicle solution comprising 500 µL of sodium chloride (0.9% NaCl) and 5 µL of ethanol (1% EtOH), respecting the animals' circadian cycles.

When 48 hours had elapsed after administration of the TAA, the animals were weighed and anesthetized with an intraperitoneal solution containing ketamine hydrochloride (95 mg/Kg) and 2% xylazine hydrochloride (8 mg/Kg). Blood was then taken by puncture of the retro-orbital plexus with a glass capillary tube and stored in test tubes with heparin to prevent coagulation.

The animals were anesthetized by anesthetic overdose, at a dosage three times larger than normal, according to the euthanasia guidelines of Brazil's National Council for Control of Animal Experimentation [21]. After confirmation of death, trichotomy and disinfection of the abdominal region was performed, followed by surgical intervention, starting with mid-ventral laparotomy, followed by removal of the liver. A liver fragment was immersed in 10% formaldehyde solution for histological examination, while another fragment was frozen for other analyses.

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### 2.3. Assessment of lipid peroxidation

After the determination of protein content in liver homogenate by the Bradford's method [23], the LPO was investigated using the thiobarbituric acid reactive substances (TBARS) technique. This technique consists of heating the homogenate with thiobarbituric acid, with consequent formation of a pink product. The coloration appears because of presence of malondialdehyde and other substances resulting from LPO in biological material.

Homogenized samples of liver were mixed with trichloroethanoic acid (10%) and thiobarbituric acid (0.67%), heated in a boiling water bath for fifteen minutes, and then cooled in ice for approximately 5 minutes. Soon after this procedure, n-butyl alcohol was added, agitated for 30 seconds in a vortex, and centrifuged for 10 minutes at 2000 rpm. The supernatant was transferred to a 96-well plate for reading at 535 nm and the results expressed as nmoles/mg protein [24].

### 2.4 Assessment of carbonyl groups in hepatic tissues

Oxidative damage to proteins was assessed by determination of the content of carbonyl groups, following the manufacturer's instructions for the Protein Carbonyl Content Assay Kit (MAK094-1KT, Sigma-Aldrich®), which is based on reaction with 2.4-dinitrophenylhydrazine (DNPH), leading to formation of stable dinitrophenyl (SDN). Proteins precipitate in response to addition of trichloroethanoic acid, which is followed by dissolution in DNPH and reading of absorbance at 375 nm [25].

#### *2.4.1 Spectrophotometric analysis of biochemical parameters*

Liver integrity was measured by the activity of three liver enzymes, i.e., aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), in the plasma using a kinetic spectrophotometric assay (Labtest®). Ammonia concentration was quantitatively determined using the animals' serum (Sigma Aldrich®, AA0100) based on the capacity to react with α-ketoglutaric acid that reduces to NADPH [26].

The activity of superoxide dismutase (SOD) was assessed by its ability to inhibit a reaction between the superoxide radical and adrenaline [27], while the activity of catalase (CAT), an antioxidant enzyme, was determined by the decomposition rate of hydrogen peroxide added to a sample [28].

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### 2.5 Analysis of inorganic elements

Identification and quantification of inorganic elements was performed by processing blood samples using the particle-induced X-ray emission (PIXE) technique [29]. These experiments were conducted in the Ion Implantation Laboratory in the physics department of the Universidade Federal do Rio Grande do Sul.

Blood samples were dried at 60° C for 48 h and then homogenates were pressed into pellets and placed in the PIXE reaction chamber. A tandem accelerator (Tandetron, 3 MV) was then used to produce a 2.0 MeV proton beam with a mean current of 5 nA at the target. Results for element concentrations were expressed in PPM [30].

### 2.6 Microscopic assessment of hepatic tissue

#### *2.6.1 Histological analysis by optical microscopy*

For the microscopic analysis, liver specimen slides were stained with hematoxylin-eosin (HE). First, samples of tissues were fixed in 10% formalin and embedded in paraffin. The paraffin blocks were then set in a microtome (Leitz® 1512) to cut three-micron slices (3µ). During the staining phase, slides were immersed in hematoxylin-eosin stains for 5 minutes each and then in a running water bath. For dehydration, structures passed through three receptacles containing absolute alcohol and two containing xylol. After this procedure, a coverslip was placed on the slide using Canada balsam or Entellan, thereby completing the preparation process.

Slides were assessed by one of the authors (blinded to group membership), using a microscope equipped with a digital camera to capture images using Image-Plus software (Media Cybernetics®, Bethesda, USA), following the histopathological criteria for SALF adopted by Lefkowitch [31]. A morphometric analysis was used to identify the severity of tissue damage based on a point-counting method using an ordinal scale as previously described [32] with the aid of ImageJ® software [33].

#### *2.6.2 Immunohistochemistry*

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Tissue samples were fixed in 10% formalin and embedded in paraffin; then, they were deparaffinized with xylol and rehydrated at different ethanol grades. Antigen retrieval was performed in a microwave oven at 100°C using citrate buffer, and the activity of endogenous peroxidase was blocked by incubating the slides in absolute MetOH containing 3% hydrogen peroxide at room temperature.

The sections were sequentially pre-incubated with 10% rabbit serum at room temperature to block possible unwanted reactions from the secondary antibody. The slides were incubated with nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein (Keap1) (Santa Cruz Biotechnology®, Santa Cruz, CA, USA), diluted at 1:200 overnight at 4º C. Then, they were washed with buffer and incubated with biotinylated anti-rabbit IgG antibody for 30 minutes at room temperature. Next, the slides were treated with EnVision® reagent and submitted to three rounds of washing with phosphate-buffered saline (PBS). The nuclei were counterstained with hematoxylin. The primary antibody was diluted in PBS containing bovine albumin as a negative control.

The results were reviewed by a pathologist who had no previous knowledge of the experimental groups through a microscope equipped with a digital camera to capture images using Image-Plus software (Media Cybernetics®, Bethesda, USA).

### *2.6.3 Ultrastructural analysis using transmission electron microscopy*

In transmission electron microscopy, images of thin sections of cells are formed by passing an electron beam through the sample so that the beam is absorbed and spread, producing contrast and an image [34].

Micrographs of the samples were obtained using a JEM-2100 electron microscope (Lab6®) with magnifications of 2000 times for the precursor and from 5000 to 10000 times for all groups after 48 hours of the experiment. The preparation of the materials for analysis followed the technique described by Tanaka and Mitsushima [35], in which fragments of the liver sample previously immersed in a fixing solution of 0.5% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer at 0.1 mol/L (pH 7.4) were placed on an adhesive carbon tape attached to a sample holder. Then, a thin layer of gold was used to improve conduction in the sample. After analysis, images were captured.

## 2.7 Statistical analysis

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The values recorded were expressed as means and standard deviations and subjected to analysis of variance (one-way ANOVA) followed by the Student-Newman-Keuls multiple-comparisons test, considering a significance level of $p < 0.05$, using GraphPad prism 6.0.

## 3 RESULTS

The analysis of enzyme activity and liver histology indicates the diagnosis of SALF. The changes resulting from oxidative stress were more pronounced in animals given only TAA, while antioxidant and hepatoprotective effects were stronger in animals treated with Mel.

### 3.4 Liver integrity

The serum levels of AST, ALT, and ALP were significantly higher in the animals given only TAA. A between-group comparison showed that Mel was able to reduce the level of AST by approximately 70%, ALT by 38%, and ALP by 40%, as shown in Table 1.

**Table 1.** Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

| Enzymes | CO | CO+Mel | TAA | TAA+Mel |
|---|---|---|---|---|
| ALT (U/L) | 82.93 (±6.99) | 83.80 (±7.02) | 168.66 (±7.68)$^{*}$ | 103.27 (±9.96)$^{\#}$ |
| AST (U/L) | 153.46 (±6.63) | 155.90 (±3.77) | 263.62 (±4.92)$^{**}$ | 78.40 (±5.50)$^{\#\#}$ |
| ALP (U/L) | 353.23 (±19.43) | 316.66 (±40.94) | 511.23 (±37.96)$^{*}$ | 243.86 (±26.56)$^{\#\#}$ |

Data are expressed as mean ± standard error. Significant increase in the TAA group in relation to the CO and CO+Mel groups: $^{*}p < 0.05$, $^{**}p < 0.001$. Significant decrease in relation to the TAA group: $^{\#}p < 0.05$, $^{\#\#}p < 0.005$.

### 3.5 Ammonia concentration

Serum ammonia levels were significantly higher in animals given TAA compared to the treatment group (TAA+Mel), which had a significant reduction (~75%), as shown in Figure 2.

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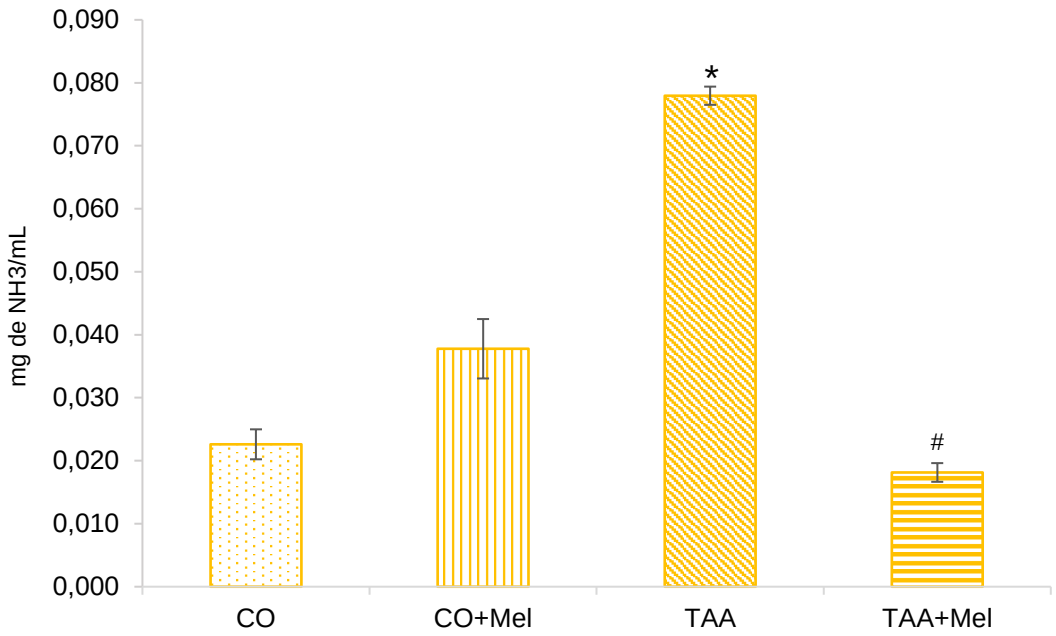


**Figure 2**. Values expressed as milligrams of ammonia (NH3) per milliliter (mL) in the four experimental groups. CO, control; CO+Mel, control plus melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. Data are expressed as mean ± standard error. *Significant increase in relation to the CO and CO+Mel groups ($p < 0.001$). #Significant decrease in relation to the TAA group ($p < 0.001$).

### 3.6 Lipid peroxidation

The results for LPO are illustrated in Figure 3. The TAA group exhibited an accentuated increase ($p < 0.01$) when compared to the CO and CO+Mel groups. In comparison, the LPO value for animals treated with melatonin (TAA+Mel) was around 85% lower, which is a highly significant finding ($p < 0.001$).

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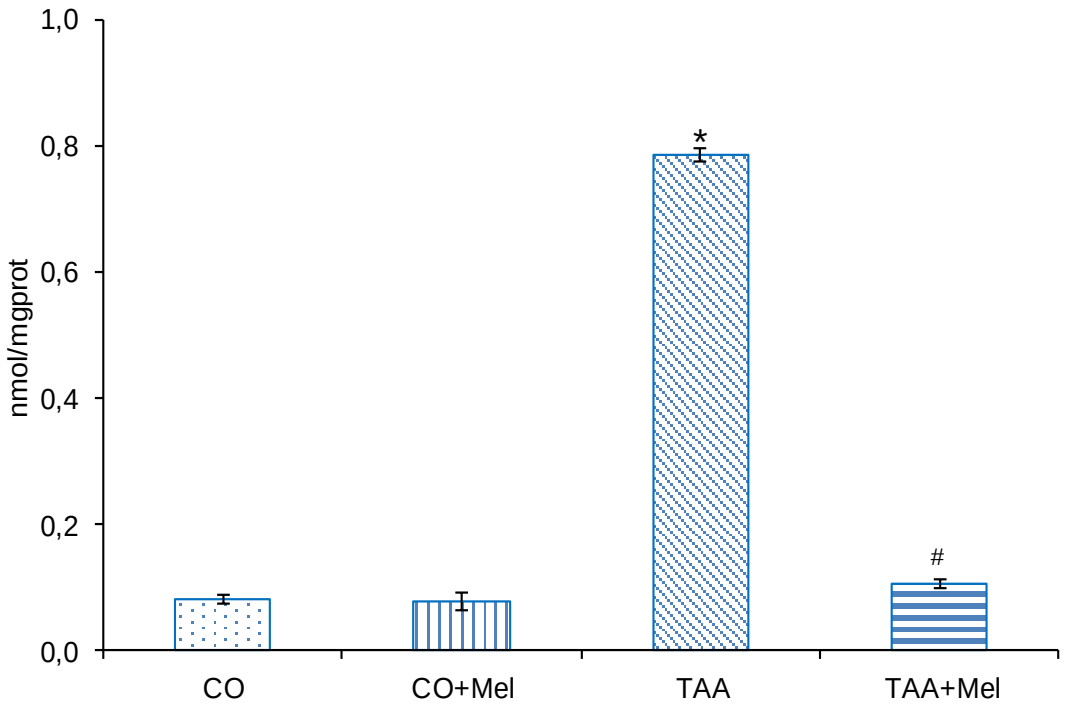


**Figure 3.** Lipid peroxidation values determined by the thiobarbituric acid reactive substances (TBARS) technique (nmol/mg prot) in the four experimental groups. CO, control; CO+Mel, control plus melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. Data are expressed as mean ± standard error. *Significant increase in relation to the CO and CO+Mel groups ($p < 0.01$). #Significant decrease in relation to the TAA group ($p < 0.001$).

### 3.7 Oxidative damage to hepatic proteins

Protein carbonylation can be used to detect and quantify oxidative changes. The results showed that concentrations of carbonyls were significantly higher in the group of animals given TAA and reduced in animals treated with Mel, as illustrated in Figure 4.

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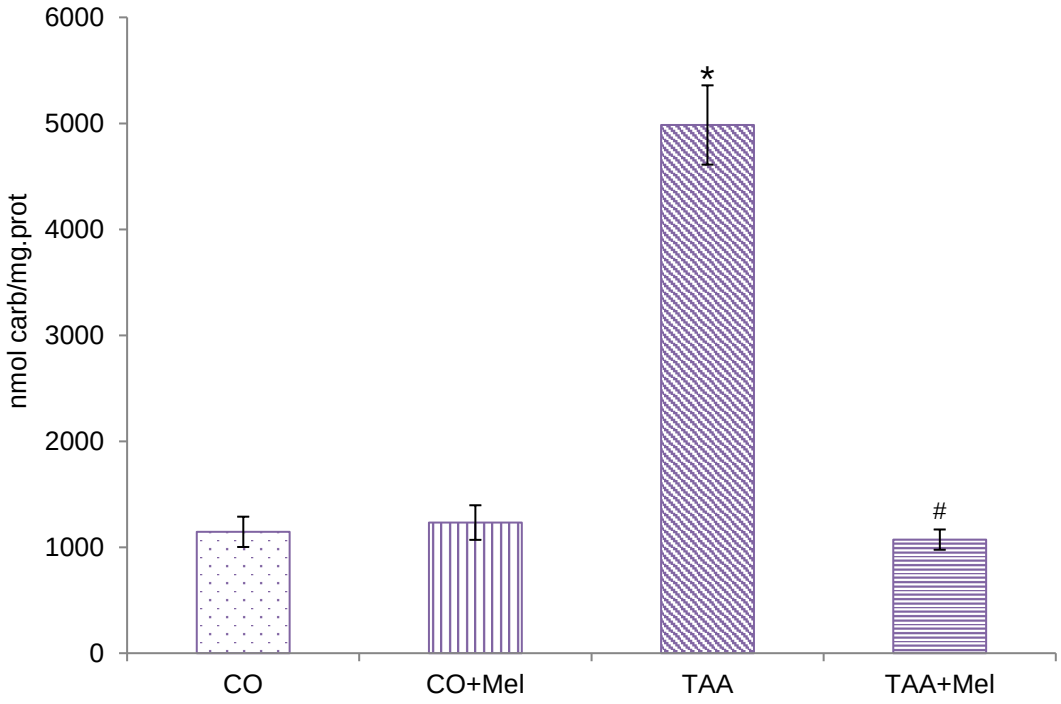


**Figure 4**. Carbonyl concentrations (nmol/carb/mg prot) in the four experimental groups. CO, control; CO+Mel, control plus melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. Data are expressed as mean ± standard error. *Significant increase in relation to the CO and CO+Mel groups ($p < 0.001$). #Significant decrease in relation to the TAA group ($p < 0.001$).

### 3.8 Superoxide dismutase and catalase enzyme activity

As illustrated in Figure 5, SOD enzyme activity was significantly higher ($p < 0.001$) in the TAA group than in the CO and CO+Mel groups. There was also a significant reduction ($p < 0.001$) in the SOD enzyme activity in the TAA+Mel group, of around 70%.

The values observed for CAT enzyme activity revealed a significant reduction ($p < 0.05$) for animals given only TAA, followed by a significant increase ($p < 0.001$) of around 70% in the treatment group (TAA+Mel), as illustrated in Figure 6.

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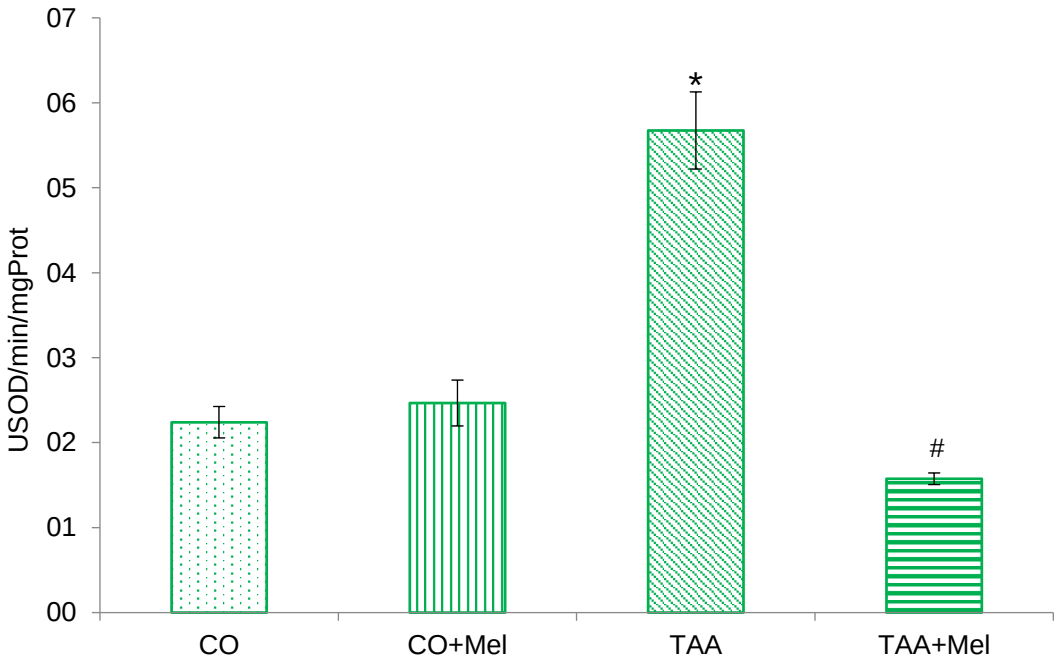


**Figure 5.** Superoxide dismutase (SOD) activity (USOD/min/mg prot) in the four experimental groups. CO, control; CO+Mel, melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. Data are expressed as mean ± standard error. *Significant increase in relation to the CO and CO+Mel groups ($p < 0.001$). #Significant decrease in relation to the TAA group ($p < 0.001$).

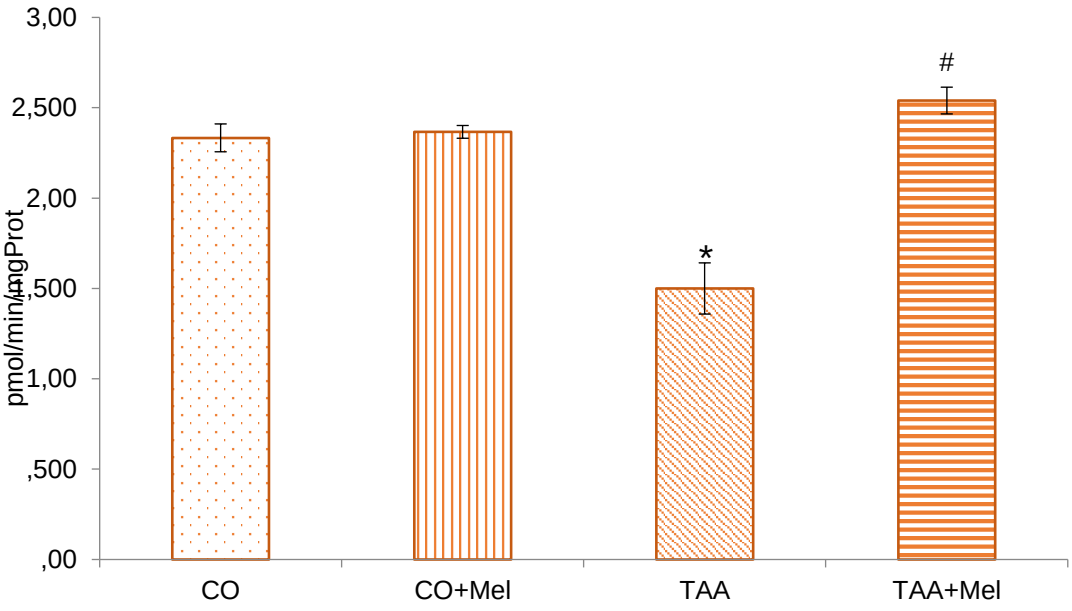


**Figure 6.** CAT enzyme activity (pmol/min/mg prot) in the four experimental groups. CO, control; CO+Mel, control plus melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. Data are expressed as mean ± standard error. *Significant decrease in relation to the CO and CO+Mel groups ($p < 0.05$). #Significant increase in relation to the TAA group ($p < 0.001$).

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### 3.6. Analysis of the inorganic elements

The inorganic elements identified are listed in Table 2. It was observed that the concentration of aluminum (Al) was five times greater in the group treated with Mel than in the TAA group. There was also a significant increase in the quantity of phosphorous (P), of approximately 10%. However, there were predominantly significant reductions in the quantities of the elements present in the samples from the treated groups (TAA+Mel), when compared with the TAA group, in particular of calcium (Ca), copper (Cu), iron (Fe), and potassium (K).

### 3.7. Relationship between superoxide dismutase activity and inorganic elements

Having observed reductions in the concentrations of certain elements and also in SOD activity in animals given Mel, correlations were calculated for the data obtained for all of the experimental groups, as illustrated in Figure 7. The results indicate a significant directly proportional relationship ($p < 0.05$) between SOD activity and the quantities of As, Ca, and Fe.

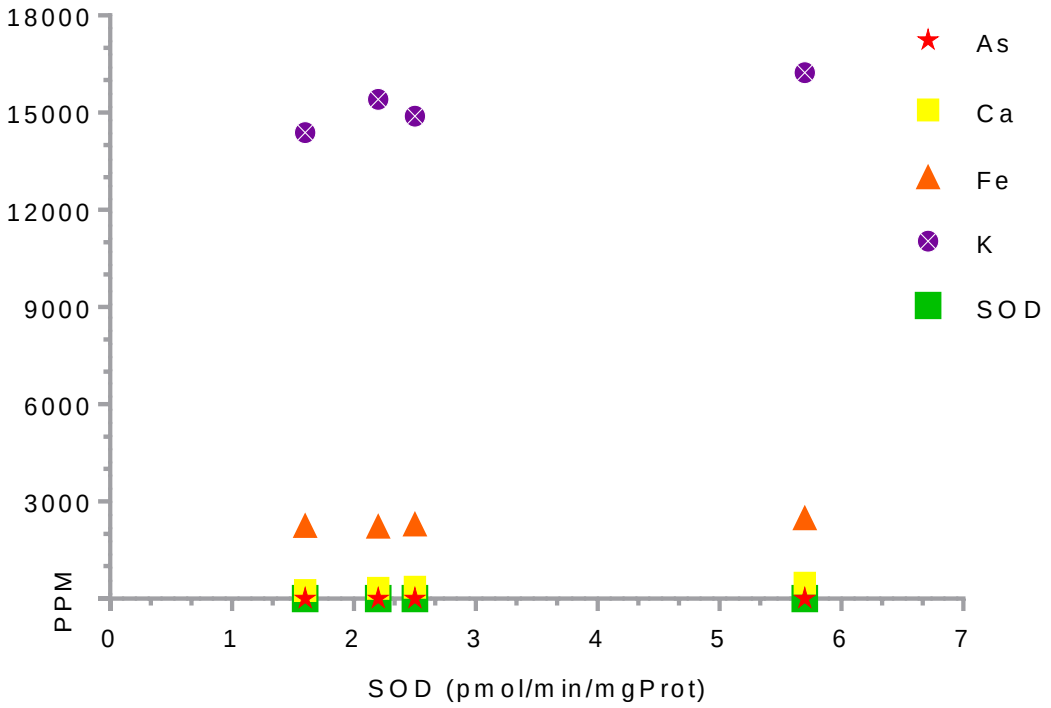


**Figure 7.** Correlations between SOD enzyme activity and inorganic elements identified. As vs. SOD (r = 0.0027, $p$ = 0.0073), Ca vs. SOD (r = 0.9722, $p$ = 0.0278), Fe vs. SOD (r = 0.9746, $p$ = 0.0254) K vs. SOD (r = 0.8992, $p$ = 0.1008). GraphPad Prism (6.0): Pearson's R Test. *Arsenic (As), calcium (Ca), iron (Fe), potassium (K), superoxide dismutase (SOD).*

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**Table 2**. Quantitative description of the inorganic elements identified.

| Element | CO | CO+Mel | TAA | TAA+Mel |
|---|---|---|---|---|
| Al | 664.55 (±45.85)$^{**}$ | 902.90 (±21.50)$^{§§}$ | 225.30 (±41.60) | 1087.00 (±24.85)$^{###}$ |
| As | 5.29 (±0.95)$^{**}$ | 6.71 (±0.78)$^{§§}$ | 10.95 (±0.54) | 4.56 (±0.96) $^{###}$ |
| Br | 34.22 (±0.16) | 28.72 (±1.46)$^{§§§}$ | 36.25 (±0.82) | 30.72 (±0.89)$^{###}$ |
| Ca | 324.93 (±19.60)$^{*}$ | 350.57 (±19.15) | 483.43 (±51.28) | 253.0 (±16.46) $^{##}$ |
| Cl | 10081.25 (±32.68) | 10651.33 (±41.47) | 9917.33 (±61.95) | 10135.0 (±64.95) |
| Cu | 5.83 (±0.51) | 2.86 (±0.18)$^{§§§}$ | 4.92 (±0.34) | 1.92 (±0.07)$^{###}$ |
| S | 5949.00 (±212.11) | 6267.33 (±139.36) | 6763.00 (±15.58) | 6741.40 (±21.21) |
| Fe | 2227.33 (±44.86)$^{***}$ | 2293.33 (±16.68)$^{§§}$ | 2491.00 (±25.54) | 2252.00 (±17.86)$^{###}$ |
| P | 3111.00 (±93.86) | 3259.33 (±64.41) | 3157.00 (±55.32) | 3454.25 (±43.44 $^{#}$ |
| K | 15412.00 (±365.38) | 14888.33 (±81.71)$^{§§}$ | 16235.00 (±119.93) | 14380.25 (±168.68)$^{###}$ |
| Mg | 203.60 (±48.80) | 161.56 (±29.92) | 236.00 (±10.28) | 209.10 (±29.87) |
| Rb | 26.11 (±2.87) | 21.17 (±2.47) | 34.25 (±1.40) | 26.89 (±1.19) |
| Si | 570.95 (±38.85) | 643.40 (±62.30) | 240.63 (±21.18) | 451.63 (±149.00) |
| Na | 10225.00 (±162.50)$^{**}$ | 10980.25 (±119.50)$^{§§§}$ | 9449.60 (±47.26) | 8723.25 (±146.19)$^{##}$ |
| Zn | 53.37 (±3.65) | 61.12 (±2.30) | 55.36 (±1.23) | 45.37 (±1.21)$^{#}$ |

Identification and concentration of inorganic elements expressed as mean (±standard error) in parts per million (PPM). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$: significant differences between the CO group and the TAA group; $^{§}p < 0.05$, $^{§§}p < 0.005$, $^{§§§}p < 0.0005$: significant differences between the CO+Mel group and the TAA group; $^{#}p < 0.05$, $^{##}p < 0.005$, $^{###}p < 0.0005$: significant differences between the TAA group and the TAA+Mel group. CO, control; CO+Mel, control plus melatonin; TAA, thioacetamide; TAA+Mel, thioacetamide plus melatonin. *Aluminum (Al), Arsenic (As), Bromine (Br), Calcium (Ca), Chlorine (Cl), Copper (Cu), Sulfur (S), Iron (Fe), Phosphorous (P), Potassium (K), Magnesium (Mg), Rubidium (Rb), Silicon (Si), Sodium (Na), Zinc (Zn)*

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### 3.8. Histological analysis of the hepatic tissues

Figure 8 contains images showing normal hepatic parenchymal structures (A and B), characterized by presence of well-defined hepatocyte cords with stained nuclei. In C, there is disorganized tissue provoked by the TAA, with extensive cell rupture and degeneration by ballooning with centrilobular necrosis.

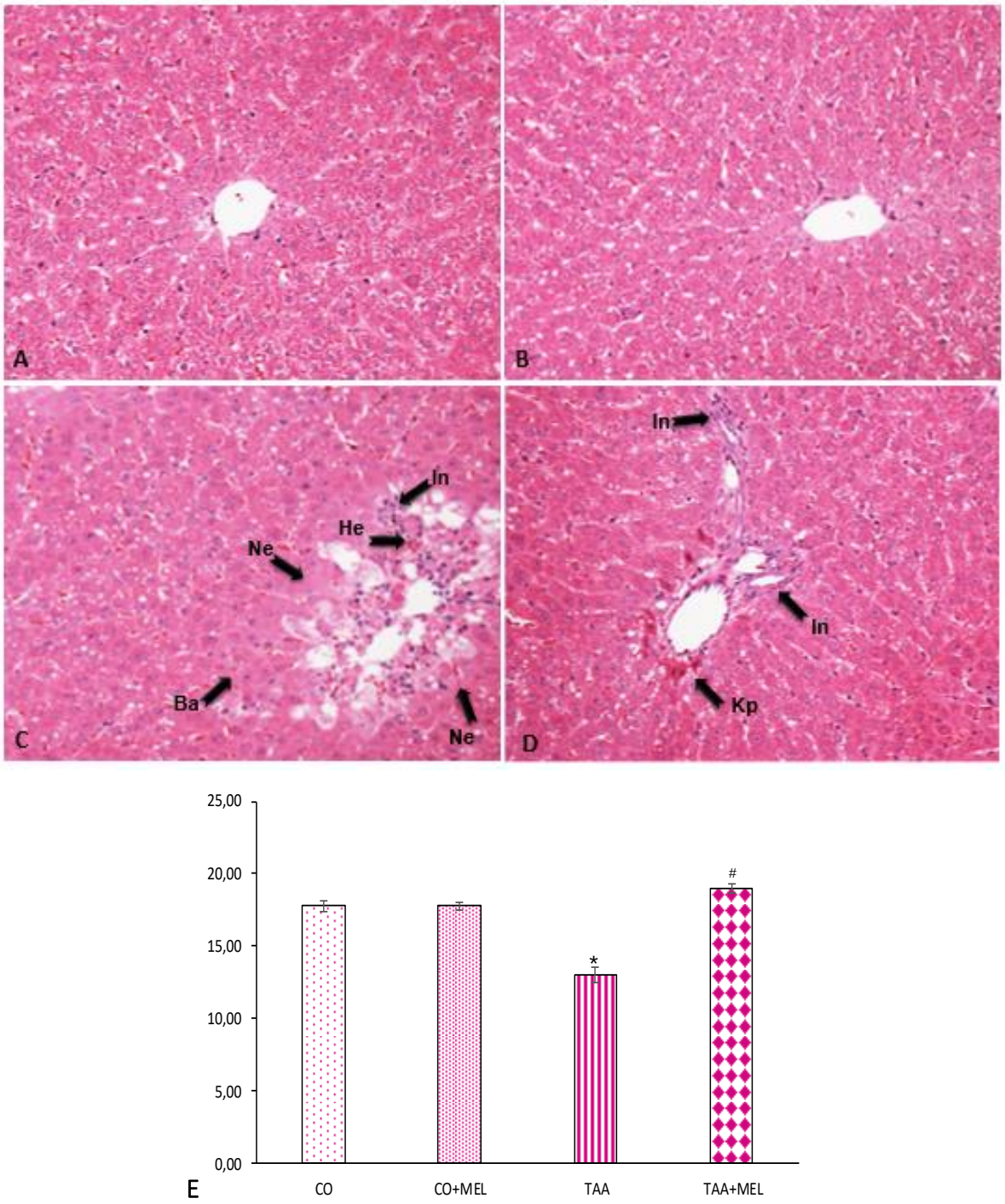


**Figure 8.** Microscopy of hepatic tissues (HE, 200x). In **A** and **B**, observe the normal architecture of the hepatic parenchyma from the CO and CO+Mel groups, respectively. Image **C** is from the TAA group, with black arrows

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indicating necrosis (Ne), inflammatory infiltrate (In), hemorrhage (He), and ballooning (Ba). In **D**, note that the specimen from the TAA+Mel group shows signs of parenchymal restructuring, with reduced manifestation of He and presence of Kupffer cells (Kp). In E, the morphometry results revealed a * significant reduction in the percentage of area occupied by cells in the TAA group, when compared to groups CO and CO+Mel, and a #significant increase in the TAA + Mel group (Image J®, *,#$p < 0.0001$).

There are many Kupffer cells, polymorphonuclear leukocytes, and other macrophage-recruiting cells. The black arrows mark disarrangement changes produced by the xenobiotic substance: necrosis (Ne) can be identified by the absence of nuclei at several points, but primarily close to the blood vessel; inflammatory infiltrates (In) indicate induced migration of leukocytes, characterizing inflammation; and there are also signs of hemorrhage (He). In the TAA+Mel group (D) the parenchymal architecture was maintained and the integrity of hepatocytes is preserved with few changes.

### 3.9. Expression of Keap1

There was a significant increase in the expression of positive pixels (E) in animals given only TAA when compared to those in the CO (A) and CO+Mel (B) groups, as shown in Figure 9. In contrast, there was also a significant decline in pixel expression ($p<0.001$) in animals treated with TAA + Mel (D).

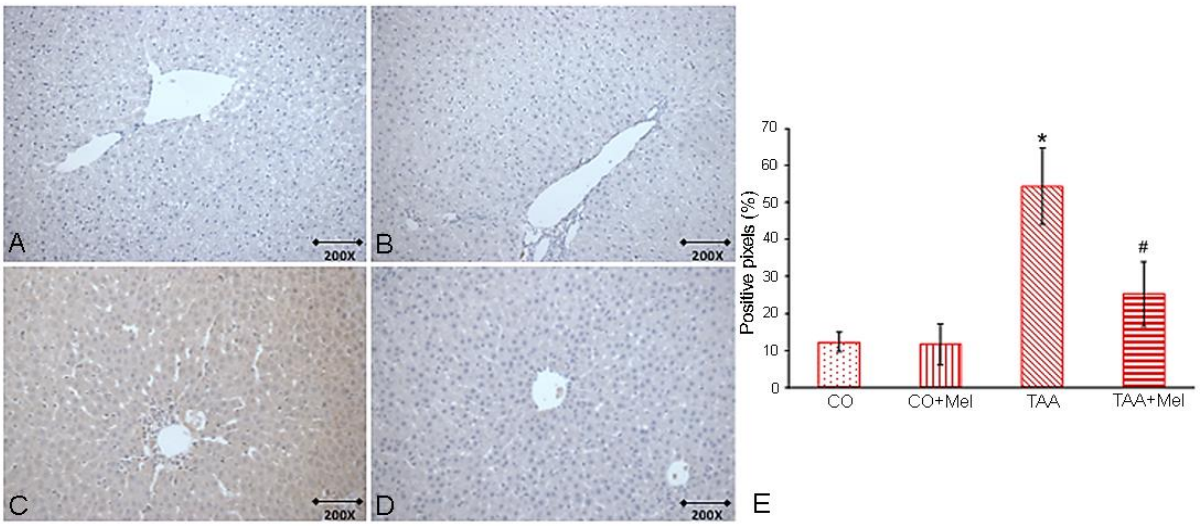


**Figure 9.** Immunohistochemical expression of Kelch-like ECH-associated protein 1 (Keap1). **A**) CO group, **B**) CO+Mel group, **C**) TAA group, **D**) TAA+Mel group, **E)** percent quantification of positive pixels. *$p < 0.001$: significant increase in relation to the CO and CO+Mel groups; #$p < 0.001$: significant reduction in relation to the TAA group (n=28).

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### 3.10. Expression of Nrf2

A significant decline ($p<0.001$) was found in positive pixels (E) in the TAA (C) group compared to the CO (A) and CO+Mel (B) groups, as shown in Figure 10. Likewise, a significant increase ($p <0.001$) was observed in animals treated with TAA + Mel (D).

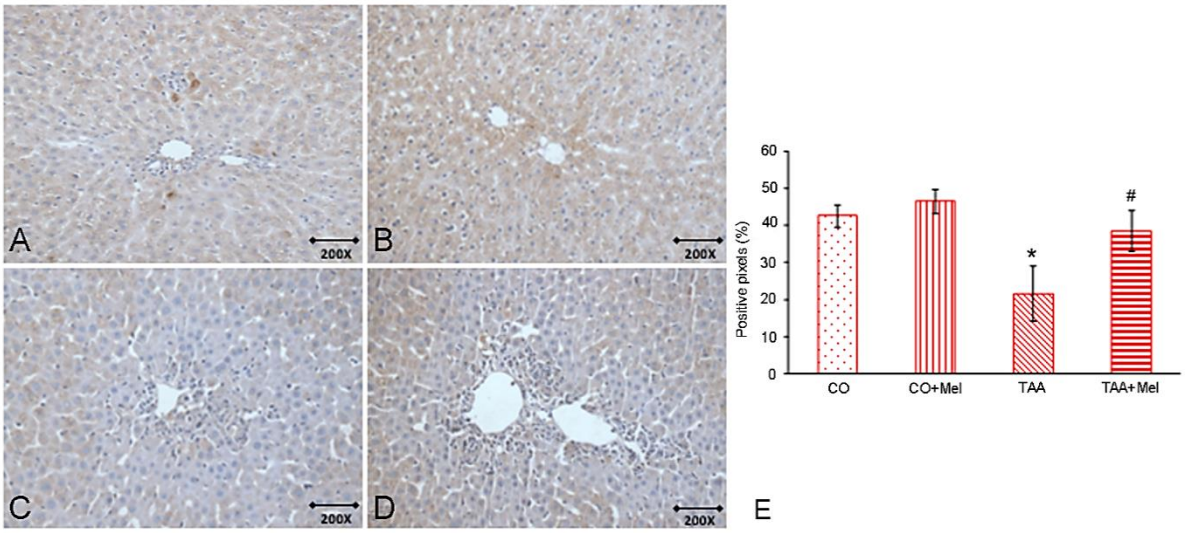


**Figure 10.** Immunohistochemical expression of nuclear factor erythroid 2-related factor 2 (Nrf2). **A**) CO group, **B**) CO+Mel group, **C**) TAA group, **D**) TAA+Mel group, **(E)** percent quantification of positive pixels. *$p < 0.001$: significant reduction in relation to the CO and CO+Mel groups; $^{\#}p < 0.001$: significant increase in relation to the TAA group (n =28).

### 3.11. Ultrastructural assessment of hepatic tissue

Normal intracellular structural components were found in the control groups (Figure 11). The nuclei were organized and enclosed by intact nuclear membranes perforated by nuclear pores. All visible cell organelles had a natural shape and size and did not appear to have structural damage capable of affecting their functions.

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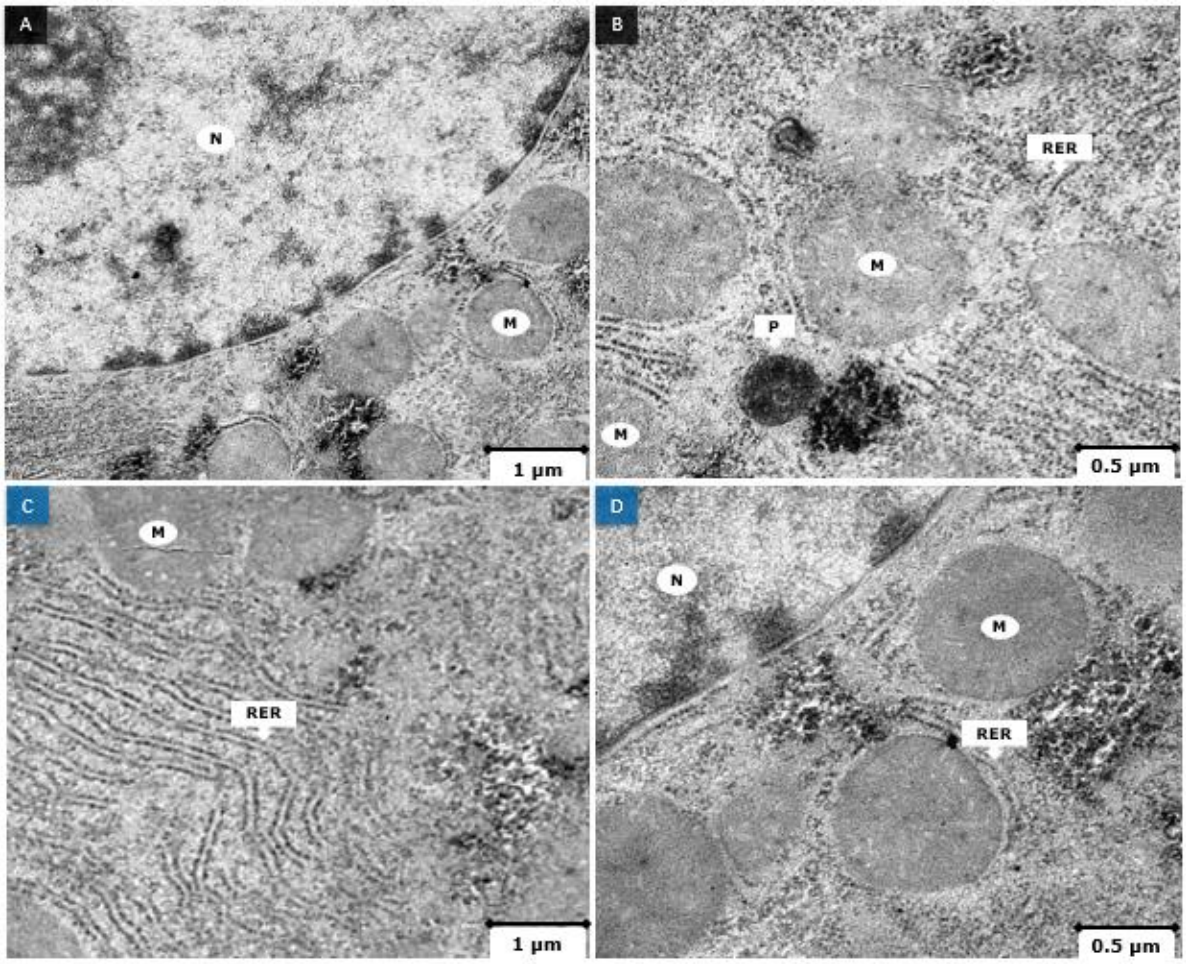


**Figure 11.** Transmission electron microscopy of hepatic tissue in the CO (**A/B**) and CO+Mel (**C/D**) groups. Hepatocytes of the animals in the control groups had normal appearance, including an intact and continuous nuclear membrane. M, mitochondrion; N, nucleus; P, peroxisome; RER, rough endoplasmic reticulum.

In Figure 12, the microelectrographs of the hepatic tissue of animals in the TAA group (E/F) revealed nuclear membranes with irregular and discontinuous contours, as well as greater peripheral heterochromatin. Noteworthy were the presence of autophagic vacuoles, lipid droplets, and damaged organelles, absence of rough endoplasmic reticulum (RER), and greater number of lysosomes and peroxisomes. For animals treated with Mel (G/H), the main findings included absence of autophagy vacuoles and preservation of cytoplasmic organelles, especially the endoplasmic reticulum. Also, there was a lower proportion of lipid droplets, and the nucleus was characterized by dense peripheral heterochromatin and an irregular nuclear membrane.

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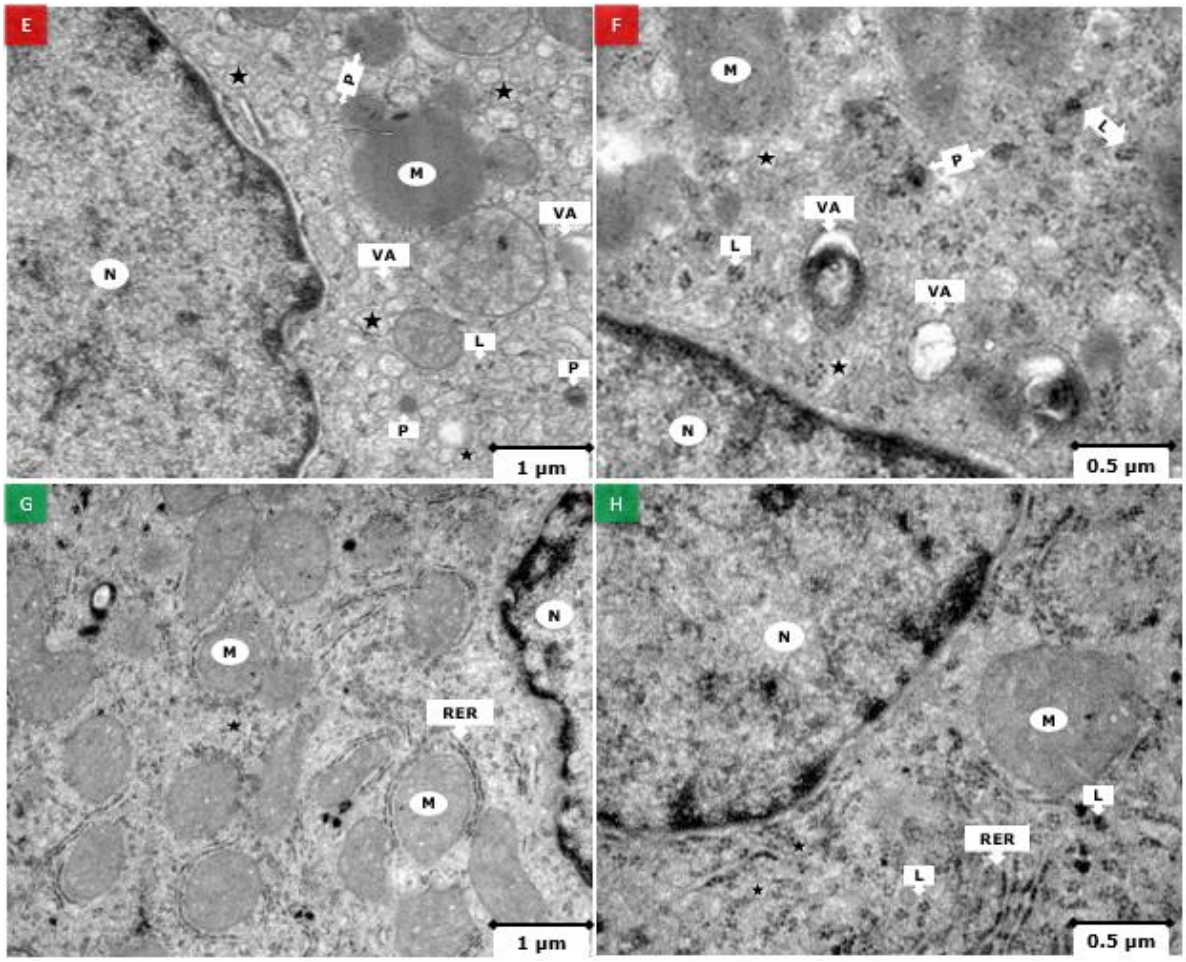


**Figure 12.** Transmission electron microscopy of hepatic tissue in the TAA (**E/F**) and TAA+Mel **(G/H)** groups. In sick animals, there was an increase in the levels of lysosomes (L) e peroxisomes (P), as well as presence of autophagic vacuoles (AV) and lipid droplets ( ). M = mitochondrion, N = nucleus, RER = rough endoplasmic reticulum.

## 4. DISCUSSION

Acute liver failure is a disorder that affects a healthy liver and manifests as a set of signs and symptoms that, in its severe form (i.e., SALF), may have rapid and fulminant progression. The importance of experimental models for understanding pathophysiologic aspects and for defining strategies for therapeutic intervention has diversified and increased over recent years [36].

The present study used TAA to induce SALF. This model is widely used in liver research because of its recognized hepatoxicity resulting from the production of ROS and the consequent LPO, increased transaminases, and centrilobular necrosis, attributed to the ability to covalently modify cell nucleophiles, lipids, and proteins [8,37,38]. Additionally, this model is recommended by the International Society for Hepatic Encephalopathy and Nitrogen

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Metabolism as it manifests acute liver dysfunction concomitantly with neuronal changes [39,40].

In the present study, a significant increase in transaminases was related to presence and greater severity of liver damage. Increased levels of AST and ALT are known to be associated with damage caused to the liver cell membrane, as there is intracellular leakage of those cytosolic enzymes [41]. Moreover, elevated ALP combined with increased concentrations of AST and ALT indicates hepatocellular dysfunctions associated with xenobiotics [42,43].

The cytoprotective effect observed in the present study was previously associated with decreased activity of transaminases and ALP due to the influence of Mel [44,45]. It was also reported in a model of TAA-induced liver fibrosis in rats treated with Mel and in the pretreatment of cyclophosphamide-induced hepatotoxicity [46]. Furthermore, similar results were found using both quercetin and glutamine as monotherapy for the same model of SALF induction used in the present study [22,47,48].

In the present study, Mel was able to reduce the serum concentration of ammonia, which is consistent with previous results [49-51]. The intraperitoneal administration of TAA in rats in a model of SALF induction is able to promote encephalopathy probably because of the increased levels of ammonia in the central nervous system [52].

As reviewed elsewhere [53], an elevated serum concentration of ammonia plays a key role in the understanding of hepatic encephalopathy, as when it passes through the blood-brain barrier, its metabolism triggers cytotoxicity. Thus, intracranial hypertension associated with a buildup of ammonia due to changes in urea metabolism by hepatocytes may occur, as well as development of hepatic encephalopathy and impairment of the neurological system [54,55]. These changes resulting from a higher brain ammonia concentration are associated with a worse prognosis in SALF; also, there are reports of neurocognitive and psychiatric disorders in survivors [56].

It is worth noting that increased serum levels of ammonia induce the production of reactive nitrogen species and, consequently, cause oxidative stress [57]. Thus, they promote damage to cell membrane proteins, contributing then to an increase in the inflammatory process and LPO.

LPO levels were more elevated in the TAA group and were reduced in both groups treated with Mel, which indicates it has a protective effect for hepatocytes. Similar results have been observed both with glutamine and quercetin for treatment of TAA-induced SALF [22,47].

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Mel was also able to reduce LPO in other animal models, such as in liver cirrhosis induced by carbon tetrachloride [58] and by bile duct ligation [59].

Increased carbonylation of liver proteins is associated with the oxidative damage provoked by TAA. The presence of the carbonyl groups aldehyde and ketones is therefore a consequence of the oxidative damage caused [24,60,61]. Melatonin was able to prevent the structural changes to proteins in hepatic tissues, once more providing evidence of its antioxidant and hepatoprotective effects in SALF.

LPO may trigger the synthesis and proliferation of hepatic stellate cells and Kupffer cells, which are more likely to be stimulated and to produce cytokines, thus increasing the inflammatory process and inducing apoptosis; in addition, there is a reduction in mitochondrial respiratory activity, leading to further production of ROS and increased oxidative stress [62].

LPO caused by administration of TAA also influences the increased levels of the superoxide anion and increased SOD activity detected in the TAA group. The findings of this study reveal a reduction in SOD activity in the treatment group, in line with a study conducted by Demirtas et al [63], using the same dose of Mel.

SOD has intracellular isoforms in the mitochondria and cytoplasm responsible for detoxification of ROS from endogenous and exogenous sources, playing an important role against oxidative stress [64]. LPO caused by the administration of TAA influences the increased levels of superoxide anion and increased SOD activity detected in the TAA groups.

SOD and CAT are the enzymes that act as cell's first line antioxidant defenses. Increased CAT activity provoked by Mel was also observed in models of salivary gland injury, aggression to testicular tissues by ionizing radiation, and multiple sclerosis [65-67]. A decreased SOD activity indicates redox imbalance and consequent oxidative cell damage, as increased levels of superoxide radicals contribute to reducing enzyme activity [66,68].

Considering the inorganic elements detected, we are aware of the complexity of understanding their distinct origins and uses in animal organisms. However, we stress that the participation of the great majority of them in cellular homeostasis and metabolism has been well-defined. Therefore, at sufficient concentrations some of these elements are considered essential, making up tissues and also serving as markers after injury. Milnerowicz et al [69] have pointed out that some metals can interact with the catalytic sites of antioxidant enzymes such as SOD and CAT.

When we analyzed models of induction of oxidative stress in animals, considering the different cell lineages and tissues affected, we found distinct and specific responses to the

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oxidative damage that selectively modulate antioxidant enzymes and also isoforms of these enzymes. This finding is of paramount importance for understanding the expression of different enzymes and signaling pathways triggered by oxidative stress [70].

In the present study, for example, the oxidative stress caused by the TAA reduced SOD activity and increased CAT activity. In contrast, Mel promoted preservation of the enzymatic activity in hepatocytes of the animals treated, similar to what was observed in the control groups. Different evidence was observed by Emamgholipour et al [67] in mononuclear peripheral blood cells, where Mel provoked increased MnSOD activity and reduced CAT activity.

We can conclude that the absence of Mn indicates that MnSOD was not present in the liver homogenate while the presence of Cu and Zn and the positive correlation between SOD activity and Fe concentration implies that the Fenton and Haber-Weiss reaction has taken place and also indicates that there is greater FeSOD activity. This analysis supports the earlier hypothesis about the specificity of the antioxidant enzyme response of each cell type, which has also been ventured by other researchers [71-73].

While FeSOD is found in cytosol, CuZnSOD is found in the intermembrane space of the mitochondria, in the nucleus, in the peroxisomes, and in extracellular spaces [74]. The CAT enzyme is present in practically all living tissues of aerobic beings, primarily located in peroxisomes, and uses Fe or Mn to break down $H_2O_2$ into $H_2O$ and $O_2$ [69].

Melatonin is capable of impeding formation of free radicals and directly sequestering $H_2O_2$, transforming it into N-acetyl-N-formyl-5-methoxykynurenamine (AFMK), which is metabolized by CAT, possibly explaining the increased CAT activity [75]. The antioxidant activity of Mel against free radicals is also due to a cascade effect it provokes, since after it has been metabolized, derivatives are generated that have scavenger effects. Examples include the metabolites cyclic 3-hydroxymelatonin (C3-OHM) and AFMK, which can protect against mitochondrial damage linked with increased levels of Ca ions [76].

These results are consistent with those of previous studies that showed the ability of Mel to modulate the activity of antioxidant enzymes at different doses, therapeutic schemes, and models of hepatotoxicity, thus supporting the benefits of Mel in modulating oxidative stress [77-80].

Histological analysis of samples from the TAA group detected disarrangement of hepatic tissue and loss of organization. This evidence may be associated with increased LPO, provoking instability in hepatocyte membranes, with consequent rupture and leakage of the

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intracellular liquid. A histopathological analysis by Shokrzadeh et al [46] revealed the efficacy of Mel for protection of hepatic cells and the same was observed by Serikov and Lyashev [81] who reported increased concentration of normal hepatocytes and reduced hepatic degeneration because of use of Mel.

In the present study, immunohistochemistry added evidence of the antioxidant and hepatoprotective effects of Mel. The hormone was able to modulate the Keap1-Nrf2 pathway by reducing the expression of Keap1 and increasing that of Nrf2.

Antioxidant response involves regulatory sequences that modulate the expression of detoxifying enzyme genes [82]. The Keap1-Nrf2 pathway is the main regulatory pathway for cytoprotection against oxidative damage caused by free radicals [83]. Changes in cysteine residues of Keap1 prevent its binding to Nrf2, which then moves in greater concentration to the nucleus, inducing the expression of transcriptional genes related to antioxidant response [84].

Thus, specific changes in the Keap1-Nrf2 pathway can explain the biological and therapeutic effects of Mel [85]. In addition, Mel has the ability to inhibit Nrf2 ubiquitination, reducing its degradation by proteasome [86]. Previous research results are consistent with those of the present study in showing that Mel can act in the Keap1-Nrf2 pathway as an antagonist to the effects related to oxidative stress [87,88].

Electron microscopy revealed that ultrastructural changes resulted from damage caused by LPO, inflammation, and loss of membrane proteins due to the oxidative stress caused by TAA, as shown elsewhere [89].

The greater number of peroxisomes found in the TAA group supports our hypothesis of potential predominance of FeSOD activity, which is natural to that organelle. Nuclear chromatin condensation and cytoplasmic vacuolization are typical signs of apoptosis [90], and vacuoles derive from ruptured mitochondria and degenerated RER [91].

Our results indicate that Mel positively influenced the functions of cell organelles by preserving them, especially the RER and mitochondria, which work together to detect the concentration of intracellular nutrients and maintain the metabolic homeostasis of hepatocytes [92]. We emphasize that the protection of cell membrane may also result from an indirect effect of Mel against oxidative stress. Also attributed to it are the modulation of the immune system and the production of endogenous substances associated with antioxidant, anti-inflammatory, and anti-apoptotic functions [93].

The antioxidant effect of Mel is attributable to the stability of the molecular structure and the low activation energy barrier against ROS [73,94]. Mel also has different metabolites

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that vary in functional groups and, therefore, have different biological properties and electron consumption capacity [95,96].

Mel-derived synthetic compounds are being studied to determine the structure-activity relationship and, thus, assess the role of different receptors and establish the pharmacodynamics for understanding the antioxidant mechanism in the several pathological conditions reported in the literature, including liver dysfunctions [97,98].

As shown in the present study, Mel has proved to be effective in protecting hepatic tissue in the induction of injuries caused by pollutants, drugs, and xenobiotics, as well as in preventing ultrastructural cell damage, necrosis, and apoptosis due to oxidative stress in models of liver disease induction [99,100].

## 5. CONCLUSIONS

In view of the findings observed in the present study, we confirm the efficacy of the experimental model of SALF development. For the treated animals, Mel was able to maintain liver integrity associated with the inflammatory process, reduce ammonia levels, and preserve hepatocytes by mitigating lipid peroxidation and membrane protein damage. In the fight against oxidative stress, Mel modulated the antioxidant response with a likely participation of different isoforms of enzymes from liver tissue and also modulated the Keap1-NrF2 pathway, which is associated with cytoprotection against oxidative damage.

Our results concerning inorganic elements and their concentrations provide data for future research to continue exploring the relationship between those and other inorganic elements for a better understanding of how oxidative stress develops in each tissue, as well as the behavior of its concentrations during interventions with exogenous antioxidant substances.

Finally, Mel supplementation reduces hepatocyte damage caused by oxidative stress by preserving the organization of liver parenchyma and maintaining the integrity of cell organelles, especially mitochondrion and endoplasmic reticulum.

## 6. ACKNOWLEDGEMENTS

We would like to express our gratitude to Brazil's National Council for Scientific and Technological Development (CNPq – National Council for Scientific and Technological Development) (CNPq) (301237/2018.02), to professor Dr. Jhonny Ferraz Diaz at the Instituto de Física da Universidade Federal de Rio Grande do Sul (RS, Brazil) and Christiane de Queiroz

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Lopes, from the Microscopy and Microanalysis Center at the Universidade Federal do Rio Grande do Sul (RS, Brazil).

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