REVIEWER ANSWERS 2979670

Very thank you for your comments and suggestions.

ANSWERING REVIEWERS 3459537

Firstly thank you for your comments and suggestions, follows the below the clarifications for the same.

- Text needs to be read out to remove some typographical and grammar mistakes. For example, in page 12, line 7 (activities of GPx and GST were...); page 17, line 23 (the sentence "when administered to Mel in BLD+Mel group" should be revised) and page 18, line 23 (describe FR at first usage).

The present article has been revised and is certified by Scientific Language revision service.

The requested corrections to pages 12 and 17 have been properly met.

Regarding the questioning linked page 18, line 23; FR abbreviation is described on page 4, line 26, where it appears pala first time.

- The abstract is appropriated but authors should describe the abbreviation "Mel" at first usage.

In the summary, Mel abbreviation has been properly described in the first use.

- Page 5, line 6. Authors should better indicate that melatonin secretion (not production) is inhibited by light.

As prompted, inhibition of Mel production was best described.

> "Melatonin (Mel, *N*-acetyl-5-methoxytryptamine) is the main product synthesized by the pineal gland, which produces Mel in a rhythmic manner, with production inhibited by light, therefore, its peak production occurs during the dark phase[15,16]."

- Page 5, 3rd paragraph. Please, include reference(s) about the investigations demonstrating the AOX potential of Mel, especially in this disease.

Regarding questioning linked the inclusion of references on page 5. The phrase was reformulated and related quotes melatonin are cited in the previous paragraph.

- Material and methods section should include the protocols for tissue homogenization and plasma extraction. Authors must indicate the zeitgeber time of melatonin administration and detailed protocols for AST and ALT determinations (or a reference describing these protocols).

The homogenized protocol and extraction of plasma were inserted.

How much to the melatonin administration time, as mentioned in item "Administration of melatonin" on page 6, the animals were treated with melatonin for 14 days, starting from the 15 days of experiment.

In relation to the protocol for determination of hepatic enzymes, they will be measured using a Liquiform Labstest® commercial kit.

- In addition, authors should also indicate where the antioxidant enzymes and GSH have been measured (plasma or liver?) and better explain the protocol for gluthatione determination. Total glutathione and oxidized glutathione were also measured?

All of the antioxidants evaluations were performed in liver homogenate. Total and oxidized glutathione enzymes have not been evaluated.

- In addition, the method for the development of immunohistochemistry (diaminobenzidine?) together with the catalogue numbers of the antibodies must be also mentioned.

The immunohistochemistry technique used is based on the reaction peroxidase + diaminoenzidina for obtaining the brownish color.

Antibodies used (mentioned in item " *Immunohistochemistry (iNOS and TNF-α)*": iNOS (SC-7271), TNF-α polyclonal antibodies (SC-52746) and secondary antibody (SC-2005).

- The uppercase letters identifying images are not correctly placed in figure 1.

The letters have been properly inserted in Figure 1.

- In the results section, it is not clearly indicated whether the measurements of liver enzyme activities, lipid peroxidation and GSH levels were taken in plasma or liver.

Evaluation of liver enzyme activity was performed in plasma. All the evaluation of oxidative stress were performed on homogenized the liver.

- Pictures from figures 4 and 5 should be better described from a histological point of view. For example, in figure 5, the BDL image is difficult to interpret taking into an account the image shown in figure 4 showing hepatocyte cords and fibrosis. Hepatocytes are not easily distinguishable in the BDL group from figure 5. By adding asterisk and/or arrows to pictures, the most important features or changes (inflammatory infiltrations, fibrosis, etc.) would be easily identified making easier image interpretation. Authors must also describe the immunostaining observed in figures 6 and 7 that seems to be restricted to the fibrotic area when using an antibody against iNOS and to the hepatocytes in the BDL group when using an antibody against TNFalpha. Furthermore, magnification or scale bars must be included in figures 6 and 7. In figure 7, authors should explain why the BDL-Mel image seems to present lower staining than the CO group while the histogram show the opposite result. - In discussion section, authors should better discuss the opposed effects observed between both antioxidant systems (SOD, CAT vs. GPx, GSH) (page 19).With relationship to Figures 4 and 5, an association between these is not possible, once the staining demonstrated in Figure 5 is specific to assess collagen deposition and a consequent fibrosis, unlike the Figure 4 where the coloring HE allows us to analyze hepatic architecture as from the staining of cell membranes.

- It was added in Figure 4 arrow indicating the presence of inflammatory infiltrate.

- With relationship to Figures 6 and 7. Immunostaining observed in figure 6 relates to inflammation, this can be related to Figure 4, where with the HE staining we can observe the presence of inflammatory infiltrate in a similar region to brownish tag present in figure 6. With respect to Figure 7, at this we can observe an extensive positive staining of brownish color, justified by the disruption of cell membranes and related to tissue destruction observed in figure 4.

- With relationship to Figure 7, the image BDL+Mel seems to present a markup lower than the represented in the histogram because it is merely a selected image to represent its group. Already the immunohistochemical expression quantification is carried out in all animals of the respective groups (n = 8), after this is carried out an average which is then represented in the histogram.

- Accordingly, since GPx activity uses reduced glutathione, authors should also explain the results in BDL group showing high GSH and GPx. Overall, this is a significant study, recommended for publication after minor revision.

In the discussion section, as discussed in the last paragraph of page 19, it is suggested that all the enzymes of the glutathione system are you increase in BDL group because they are acting in an attempt to minimize oxidative damage resulting from the disease. It is believed that, in addition to oxidative stress, the own activation of GPx with a consequent consumption of GSH is also a proponent factor for the increase of this.

ANSWERS REVIEWER 3459583

Firstly thank you for your comments and suggestions, follows the below the clarifications for the same.

IN ABSTRACT:

- In Abstract: Write LPO in full In core tip.

Write LPO in full

- I would remove the part on melatonin, and rather say "Melatonin provides hepatic-protection in the experimental model of bile duct ligation" or something similar to this.

Phrase reworded.

> *"Mel (main product of the pineal gland) acts providing hepatic-protection in the experimental model of bile duct ligation."*

In the introduction:

- Remove "is an organ that"

Removed "is an organ that"

- **This part of the sentence "several vital functions, including direct synthesis and metabolism of many substances" is too vague (please rephrase)**

Phrase reworded.

> *"The liver has a complex structure, it allows plays a key role in operation and maintenance of several vital functions of the organism, including synthesis activity and excretion of substances."*

- Add "the" before "ability to synthesize" (in paragraph one)

Added "*the*" before "*ability to synthesize*".

- Move the following paragraph "Cirrhosis of the liver represents the most advanced stage of fibrosis, in which there is evident loss of structure of the hepatic arenchyma. It is directly associated with development of septa and fibrotic nodules, changes in hepatic blood flow, and high risk of liver failure [4]" and place it after the sentence "Occurrence of these changes may lead to the development of liver fibrosis, which, in turn, can lead to secondary biliary cirrhosis [5]"

Restructured paragraph.

> *"Obstruction of the biliary tract is a congestive process that leads to numerous changes, such as ductular proliferation, stellate cell activation, and accumulation of ECM in the space of Disse. Occurrence of these changes may lead to the development of liver fibrosis, which, in turn, can lead to secondary biliary cirrhosis[4]. Cirrhosis of the liver represents the most advanced stage of fibrosis, in which there is evident loss of structure of the hepatic parenchyma. It is directly associated with development of septa and fibrotic nodules, changes in hepatic blood flow, and high risk of liver failure[5]."*

- Insert references in the following places "As cirrhosis constitutes a major public health problem (REFERENCE)" and "Prolonged obstruction of the bile duct in rats is an experimental model for induction of secondary biliary cirrhosis (REFERENCE)"

References inserted:

> *"As cirrhosis constitutes a major public health problem[7]"*
>
> *"Prolonged obstruction of the bile duct in rats is an experimental model for induction of secondary biliary cirrhosis[10].*

- **Split the following sentence into two part, it's too long: See how I adapted the sentence here "As cirrhosis constitutes a major public health problem, much research is being conducted to develop and test different substances that could be used in its treatment. The aims of such substances are to improve quality of life, increase survival, slow disease progression, and possibly, mitigate the damage caused by formation of free radicals (FRs) [7,8]"**

Phrase rephrased:

> *"As cirrhosis constitutes a major public health problem[7], much research is being conducted to develop and test different substances that could be used in its treatment. The objective of such substances aims to improve quality of life, increasing survival, slowing disease*

progression, and, possibly, mitigating the damage caused by formation of ROS and free radicals (FR)[8,9]."

- **Remove the part "and insulin resistance-related effects"; it is irrelevant for your paper.**

Removed (*and insulin resistance-related effects*)

"Several effects have been attributed to Mel, including antioxidant capacity, as well as anti-inflammatory and immunomodulatory[18-21]."

- Remove the abbreviation "AOX" from the whole manuscript please and write in full.

Abbreviation AOX removed of the manuscript.

- I would rewrite the following paragraph "Taking into account the high prevalence of this disease and the evidence for an AOX potential of Mel demonstrated in several investigations, the present study assessed the possible antioxidant effect of Mel in experimental cholestatic liver fibrosis induced by bile duct ligation in rats" and replace it with "There is an existing link between cirrhosis, inflammation and oxidative stress and treatments are required to protect the liver against damage. Therefore, this present study investigated whether melatonin (an anti-inflammatory agent and antioxidant) would afford hepatic-protection in a model of cirrhosis".

Paragraph restructured:

"There is an existing important link between cirrhosis, inflammation and oxidative stress, in this sense treatments are required to protect the liver against these damage. Therefore, this present study investigated whether melatonin (an anti-inflammatory agent and antioxidant) would afford hepatic-protection in a experimental model of cirrhosis."

IN MATERIALS & METHODS:

- "animal" instead of "animals"

"All animal procedures were conducted in accordance with the recommendations of the Health Research Ethics Committee of the Research and Graduate Studies Group (GPPG) at Hospital de Cl ńicas de Porto Alegre (HCPA), Brazil (approval number 14-0474), and as recommended in the Guide for the Care and Use of Laboratory Animals[22,23]."

- Rewrite the following part so that it is the same as in your abstract "The sample comprised 32 male Wistar rats (mean weight 300 g), which were allocated across four groups: control (CO, n=8), Mel-treated control (CO+Mel, n=8), bile duct ligation (BDL, n=8), and Mel-treated bile duct ligation (BDL+Mel, n=8)", "two knots made using 3.0 silk".

In Animals, phrase rephrased:

"The sample comprised male Wistar rats (n=32, weight ±300 g) were allocated across four groups: CO (sham BDL), BDL (BDL surgery), CO+Mel (sham BDL and Mel administration) and BDL+Mel (BDL surgery and Mel administration)."

" two knots made with 3-0 silk thread "

- Under the heading: “Liver enzyme activity” – Your first sentence should start with “Activity” and not “levels”. Also under this heading, you need references after the words “kinetic method” and “colorimetric method”

In “Liver enzyme activity": About the colorimetric method and kinetic, them were performed by the biochemistry laboratory of the HCPA using a Liquiform Labstest® commercial kit.

- **Under the heading: “Hepatosomatic and splenosomatic indices” do you mean “HSR = liver weight (g)”? Also, replace the words “mouse” with “rat”**

In Hepatosomatic and splenosomatic indices; the HSR is calculated as the percentage of total organ weight (liver), divided by the body weight of the animal, HSI = liver weight (g) / rat weight (g) × 100.

The mouse words were replaced by rat.

“HSI = liver weight (g) / rat weight (g) × 100; SSI = spleen weight (g) / rat weight (g) × 100[26].”

- Under the heading: Lipid peroxidation” to what temperature was the sample heated? 95 degrees Celsius? - Was your TBARS not perhaps expressed as “nmol malonic dealdehyde/mg protein”?

In Lipid peroxidation; heated at 100 ℃ in a water bath for 15 minutes, resultes expressed in nmol/mg protein.

- Under the heading: Histological analysis; with what was the 10% formalin buffered?

In Histological analysis; The 10% formalin is obtained from the absolute formalin by dilution with water.

IN RESULTS:

- In results section: - Under your results section and the heading “liver enzyme activities” – Please double those percentage increases/decreases in AST and ALT. It is not increased by 379%, but rather 337% and not 72% reduced but 308%. You have to double all of these percentages.

Can not understand their placement. In principle, all calculations were reviewed and are correct.

Following example:

AST (CO vs LDB)

425,8 U/L (BDL) x 100 % (CO)= 42580

42580 / 88,8 U/L (CO)= 479,5 %

479,5 % (BDL) – 100 % (CO)= INCREASING OF 379 % MORE THAN THE CO.

IN DISCUSSION:

- In the second paragraph, line 4: Start the sentence with "Our findings also demonstrated that administration of Mel to animals".

> *"Our findings also demonstrated that administration of Mel to animals with cirrhosis induced by BDL reduced the liver damage caused by duct ligation."*