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# Melatonin Supplementation Alleviates Free Radical Load, NF-κB, Cox-2 and IL-1β-Mediated Inflammatory Responses of the Liver of Cisplatin-treated Golden Hamster *Mesocricetus auratus*

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## Abstract

Cisplatin is a chemotherapeutic drug which frequently induces hepato- and renal toxicities. Cisplatin-induced hepatic damage is an area less investigated compared to renal damage. In the present study we investigated the hepatic damage caused by cisplatin and its possible protection by the hormone melatonin. Adult male golden hamster *Mesocricetus auratus* (≥ 2 months of age, and ± 100 g bw) were randomly divided into four groups (n=5)- Group I- control (injected with normal saline), group II- cisplatin (single dose of 15 mg/kg bw, *ip*), group III- melatonin (100 ug/100 g bw *ip* for 4 days) and group IV- Mel pretreatment followed by cisplatin at the above-said doses. The animals were euthanized 48 hr after the last dose. Liver was dissected out for analysis (histology, antioxidant profile, NF-κB, IL-1β, Cox-2, Hemeoxygenase-I and Nrf2). Cisplatin treatment induced steatohepatitis-like changes in the liver, elevated TBARS and suppressed antioxidant profiles. Further, the expression of NF-κB, IL-1β, Cox-2, and Hemeoxygenase-I were increased and the expression of Nrf2 was decreased suggesting inflammatory damage to liver. Pre-treatment of melatonin reduced the cisplatin-mediated hepatic pro-oxidant/antioxidant balance and inflammatory responses. Therefore, melatonin pretreatment might be a supportive approach in cancer therapy as it negates some of the damaging effects of cisplatin on liver to an extent without interfering with its chemotherapeutic attributes.

**Keywords:** Cisplatin, Inflammation, Liver, Melatonin, Oxidative Damage

## 1. Introduction

*Cis*-diamminedichloroplatinum or cisplatin is an inorganic platinum-based compound which has been proved to be very effective in treating cancer, one of the most prevalent and dreaded diseases of this century. Cisplatin acts on cancer cells by inducing DNA adduct formation which in turn leads to faulty transcription, cell cycle arrest and eventually cell death by apoptosis[1]. Cisplatin is utilized for the treatment of different types of cancers including breast, testicular, ovarian, lung, bladder, etc. However, the explicit anti-neoplastic activity of this platinum compound comes with a cost, i.e., the deleterious side-effects such as nephrotoxicity, gastrointestinal toxicity, neurotoxicity and hepatotoxicity[2]. Epidemiological data suggests that almost 25-35% of patients suffer of renal anomalies following a single dose of cisplatin and this is alarming and hence discourages the use of this drug for the cancer treatment[3]. Oxidative stress is the shift in balance between pro- and antioxidants in the body and is associated with

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almost all kinds of pathological conditions. Cisplatin induces oxidative stress which proves beneficial in killing cancer cells but simultaneously this situation also makes the healthy tissues susceptible to Reactive Oxygen Species (ROS)-mediated damages[4]. Most of the reports regarding the negative effects of cisplatin emphasize renal pathologies, and suggest that cisplatin increases the lipid peroxidation in the kidney and inhibits the antioxidative defense[5]. However, the reports on other physiologically important organs (liver, pancreas and gonads) are scanty. Therefore, in the present study we choose to investigate the damage caused by cisplatin to the liver and the possible mechanism by which these damages are produced.

Our study also involves the use of the hormone melatonin, known for anti-oxidant and anti-inflammatory properties, as a possible hepato-protective agent that might ameliorate the cisplatin-mediated hepatic damages. Melatonin has amazed the scientific world with a wide variety of physiological functions. It is a well-established antioxidant and has been scientifically proven to be more effective than other antioxidants[6]. Melatonin also possesses anti-neoplastic property[7,8]. But it does not produce side-effects even at high concentrations[9]. Melatonin treatment ameliorates the side effects, such as immune response and thrombocytopenia, of chemotherapy[10]. Therefore, keeping the above-mentioned properties of melatonin in mind we hypothesized that melatonin might be helpful in neutralizing the damaging effects of cisplatin which are primarily ROS-mediated. Moreover, the anti-cancer attribute of melatonin might prove to be an added advantage in the case of a combinatorial therapy with cisplatin wherein the two compounds shall not interfere with each other's functionality. Therefore, we examined if melatonin would be an effective substance in counteracting the ROS-mediated damaging effects of cisplatin on the liver. Hence, we studied the oxidative hepatic damages that are induced by cisplatin and the protective effects of melatonin.

# 2. Materials and Methods

All the experiments were conducted in accordance with institutional practice and within the framework of Institutional Animal Ethics Committee under the purview of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Approval No.- BHU/DoZ/IAEC/2018-19/052).

## 2.1 Animals and Maintenance

Adult male golden hamsters, *Mesocricetus auratus* (weighing 100 ± 10 g), were maintained under constant temperature (25 ± 2 °C) and photoperiodic conditions (critical photoperiod; 12.5 hours of light; 11.5 hours of darkness) in commercial polypropylene cages (475 × 350 × 200 mm) in the animal house and were provided with commercial food pellets and water *ad libitum*.

## 2.2 Drugs and Treatment

Cisplatin solution (50 mg in 0.9% w/v normal saline, Cipla House, Mumbai, India) was obtained from local pharmaceutical outlets, and further diluted in normal saline to obtain the desired dose for administration. Melatonin was purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA) and was first dissolved in a few drops of ethanol and then diluted with normal saline to make the desired concentration.

## 2.3 Experimental Design

Twenty adult healthy male golden hamsters were randomly divided into four groups (n = 5 per group). Animals in group I formed the untreated control and received normal saline through intra-peritoneal (*ip*) route. Animals in group II were administered a single *ip* dose of cisplatin (15 mg/kg body wt). Animals in group III were administered *ip* melatonin (100 µg/100 g body wt/day) for 4 days. Animals in group IV were pre-treated with melatonin, followed by a single dose of cisplatin on day 5, all at the doses as above.

At the end of the experiment i.e., 48 h after the last injection, the animals were weighed and sacrificed under aseptic conditions under deep ether anesthesia. Blood was collected in falcon tubes by cardiac puncture and the serum was isolated and stored at -80$^0$C for further analyses. The liver was dissected out, washed in chilled PBS and a part was fixed for 24 h in Neutral Buffered Formalin (NBF) for histology while the remaining tissue was homogenized in homogenizing buffer. The homogenates were centrifuged at 12000 x g for 25 min and the supernatant was collected and stored at -80$^0$C in a deep freezer for biochemical analyses and western blotting.

## 2.4 Determination of Serum Alanine Aminotransferase (ALT), Serum Glutamate Oxaloacetate Transaminase (AST) and Alkaline Phosphatase (AP) activities

Serum ALT, AST and ALP activities were determined using commercially available colorimetric kits (Arkray Healthcare and Avecon Healthcare Pvt Ltd, India) following the respective manufacturer's protocol. The serum samples were thawed and processed with different reagents provided in the kits and the absorbance was read at 510 nm in a UV-Vis spectrophotometer. The values were calculated as per manufacturer's calculation protocol.

## 2.5 Estimation of Serum Bilirubin Level

Serum bilirubin (direct) was estimated using commercially available colorimetric kits (Span Diagnostics Ltd., India) following the manufacturer's protocol. The serum samples were thawed and mixed with the different reagents provided in the kits and ultimately the absorbance was read at 546 nm in a UV-Vis spectrophotometer. The values were calculated as per manufacturer's calculation protocol.

## 2.6 Histology of Liver

The portion of the liver fixed in NBF was randomly cut into smaller pieces and kept in fresh NBF for 24h to ensure proper fixation. The tissue pieces were then processed for paraffin embedding. The blocks were cut into 7 µm thick sections in a semi-automatic microtome (Leica, Wetzlar, Germany). The sections were spread on clean oil free glass slides pre-coated with 1% gelatin. The sections were deparaffinized and stained in hematoxylin and eosin and mounted in DPX mountant. The slides were viewed in a Nikon E 200 (Japan) light microscope and documented.

## 2.7 Lipid Peroxidation Assay by TBARS Level Estimation

Fresh liver tissue was used to prepare 10% tissue homogenate in 20 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 3000 x *g* for 15 min at 4°C and supernatant was subjected to Thio-BArbituric acid (TBA) assay by mixing it with 8.1% SDS, 20% acetic acid, and 0.8% TBA and boiling for 1 h at 95°C. The reaction mixture was immediately cooled in running water and vigorously shaken with n-butanol and pyridine reagent (15:1) and centrifuged for 10 min at 1500 x *g*[11]. The absorbance of the upper phase was measured at 534 nm. LPO was expressed as TBARS in nmol/g tissue wt.

## 2.8 Superoxide Dismutase Activity Assay

Fresh liver tissue was used to prepare 10% homogenate in 150 mM Phosphate Buffered Saline (PBS, pH 7.4) and centrifuged at 12000 x *g* for 30 min at 4°C. The supernatant was again centrifuged at 12000 x *g* for 60 min at 4°C and then processed for enzymatic activity based on the modified spectrophotometric method using nitrite formation by superoxide radicals[12]. The tissue extract (0.1 mL) was added to 1.4 mL of reaction mixture comprising of 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride, and 50 mM EDTA followed by a brief pre-incubation at 37°C for 5 min. Next, 0.8 mL of riboflavin was added to all samples along with control containing buffer instead of sample and then exposed to two 20 W fluorescent lamps fitted parallel to each other in an aluminium foil coated wooden box. After 10 min of exposure, 1 mL of freshly prepared Greiss reagent (1% sulphanilamide, 5% orthophosphoric acid, 0.1% N-1-napthylethylenediamine dihydrochloride) was added and absorbance of the color formed was read at 543 nm. One unit of enzyme activity is defined as the amount of SOD that inhibits 50% of nitrite formation under assay condition.

## 2.9 Indirect Catalase Activity Assay

Liver homogenate was processed for the assay in a reaction mixture comprising 0.8 mM $H_2O_2$, PBS and potassium dichromate in glacial acetic acid. The reaction was stopped by heating in a water bath for 10 min and the OD was read at 570 nm and the decrease in $H_2O_2$ content was calculated. The decrease of $H_2O_2$ by the enzyme present in the liver extract was depicted as catalase activity. The standard curve was calibrated with varying concentrations of 0.2 mM $H_2O_2$ in PBS[13].

## 2.10 Western Blot Analysis of Nrf2, HO-I, NF-κB, Cox-2 and IL-1β

Western blot analysis was performed following the method of Goswami and Haldar[14]. The liver was dissected out and homogenized in RIPA buffer [1% (v/v) Igel CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS) in Phosphate-Buffered Saline

(PBS) containing aprotonin and sodium orthovanadate)]. Aliquots containing 100 µg protein were resolved by 12% (w/v) SDS-polyacrylamide gel electrophoresis, followed by electro-transfer (BioRad, Trans-Blot Turbo) onto a nitrocellulose (BioRad, USA) membrane. Immuno-detection was carried out using the respective primary antibodies as below:

1. Nrf2 – ab 31163 AbCam Biotechnology Company, Cambridge, UK
2. Hemeoxygenase I (HO-I) – ab13243 (SB) AbCam Biotechnology Company, Cambridge, UK
3. NF-κB - sc 1017409 Santa Cruz Biotech, USA
4. Cyclooxygenase-2 (Cox-2) – PA517614 Thermo Fisher Scientific, Waltham, USA
5. IL1β – sc 12742 Santa Cruz Biotech, USA

After the incubation, the membranes were washed and further incubated with HRP-conjugated secondary antibody (Goat anti-rabbit IgG-HRP for Nrf-2, HO-I, NfκB, Cox-2 and Mouse Anti-armenian hamster IgG-HRP for IL1β). The expression was detected using Enhanced Chemi-Luminescence (ECL) detection system (Clarity, Biorad, USA). The detected film was quantified for optical density using Scion Image Analysis software. The ratio of density was calculated with β-actin (Sigma–Aldrich) as loading control, and expressed as % relative control value. The membranes were incubated in anti-β-actin primary antibody (1:2000, Sigma–Aldrich) and immuno-detection was carried out with anti-mouse IgG-HRP secondary antibody (1:4000) followed by ECL.

### 2.11 Statistical Analysis

Statistical analysis of the data was performed with one-way ANOVA with the help of SPSS Statistics 17.0 software programme followed by Tukey's HSD post hoc test for comparisons of group means. The data were presented as the means ± SEM. The differences were considered significant when $p<0.05$.

## 3. Results

### 3.1 Body Weight

The body weight of the animals decreased following cisplatin-alone injection (Figure 2). However, there was no change in body weight in the other groups.

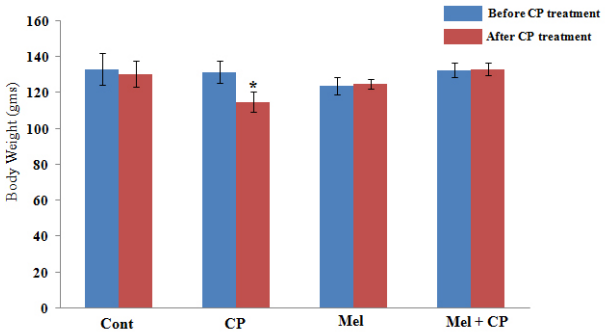


**Figure 1.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injection (15 mg/kg bw) on the body weight of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. * p<0.05 Cont vs. CP.

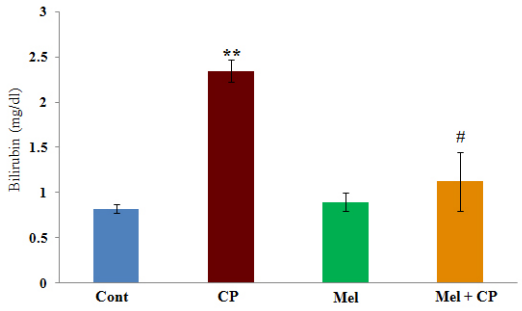


**Figure 2.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injection (15 mg/kg bw) on plasma bilirubin level of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

### 3.2 Serum Bilirubin Levels and ALT, AST and ALP activities

Cisplatin treatment caused a significant increase ($p<0.01$) in the serum bilirubin levels of the golden hamsters while melatonin treatment had no significant effect on the bilirubin levels when compared to the control (Figure 3). Melatonin pre-treated animals showed significant reduction in the serum bilirubin levels when compared with the cisplatin alone group ($p<0.01$). The activity of

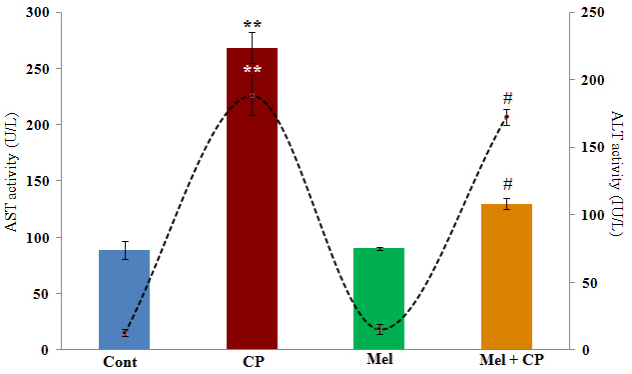


**Figure 3.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injection (15 mg/kg bw) on plasma Aspartate aminotransaminase (AST) and Alanine aminotransaminase (ALT) activities of male golden hamster *Mesocricetus auratus*. Plasma ALT activity is expressed as dashed line in secondary axis. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

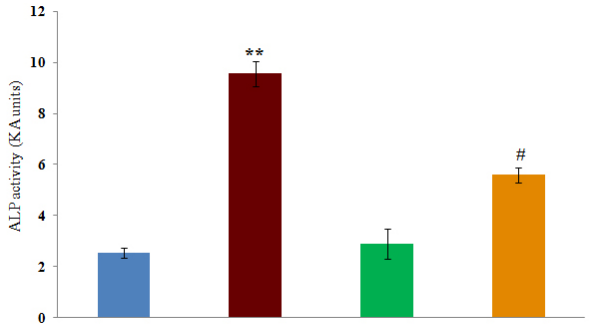


**Figure 4.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injection (15 mg/kg bw) on plasma Alkaline phosphatase (ALP) activity of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

the marker enzymes for healthy liver functionality (ALT, AST and ALP) also increased significantly ($p<0.01$) after injection of cisplatin when compared to control (Figure 4 & 5). Melatonin injections alone showed no significant effect on the activity of the liver enzymes. Interestingly, the animals pre-treated with melatonin followed by cisplatin injection depicted significant reduction ($p<0.01$) in the activity of ALT, AST and ALP, respectively.

## 3.3 General Histology of the Liver

The control and melatonin-treated animals depicted normal liver histology (Figure 5A, B, E & F) with polyhedral healthy hepatocytes arranged as cords. The hepatocytes had granular cytoplasm and a prominent central nucleus. The cisplatin treatment (Figure 5C & D) induced pathological changes in the liver with dilation of the central vein and sinusoids along with disrupted arrangement of the hepatocytes. There was marked increase of inflammatory cells. The hepatocytes showed necrosis, binucleation and increased vacuolation of the cytoplasm. Some of the hepatocytes also showed karyorrhexis. The animals pre-treated with melatonin followed by cisplatin injections presented marked improvement in the liver histology and was almost like that observed in the control animals (Figure 5G & H).

## 3.4 Oxidative Stress Parameters Assayed in Liver Homogenate (TBARS, SOD and Catalase)

The accumulation of free radicals and induction of oxidative stress was evidenced by the significant increase ($p<0.01$) in the TBARS level in the liver of the cisplatin-treated animals whereas melatonin treatment alone decreased the TBARS in the liver of golden hamsters (Figure 6). Similarly, the enzymes SOD and Catalase showed significantly decreased ($p<0.01$) activities in the liver of cisplatin-treated animals (Figure 7 & 8). Melatonin treatment-alone increased the SOD activity but had no significant effect on the catalase activity in the liver. The animals which received the melatonin pre-treatment before cisplatin depicted significant decline in the TBARS level in the liver and also showed an increase in the activity of SOD and catalase when compared to the cisplatin-only treated group ($p<0.01$).

## 3.5 Expression Patterns of Nrf-2, HO-I, NfκB, Cox-2 and IL-1β in Liver Homogenates

The expression of Nrf-2 declined significantly ($p<0.01$) in the livers of the animals treated with cisplatin when compared with the control (Figure 9a). Melatonin pre-treatment to cisplatin produced significant positive effect

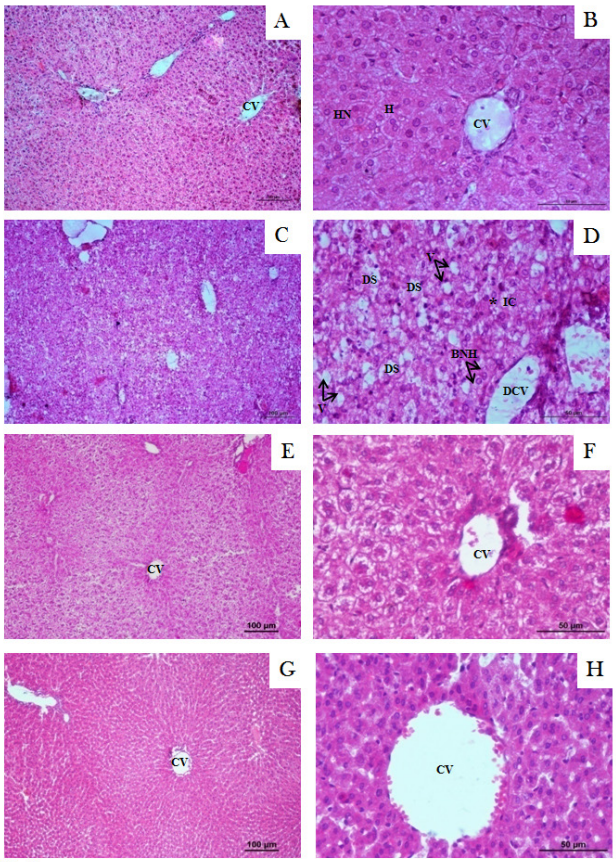


Fig. 5

**Figure 5.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injections (15 mg/kg bw) on liver histo-architecture depicted by hematoxylin and eosin staining. A & B – Control group showing normal histo-architecture of hepatocytes (H), hepatocyte nuclei (HN) and central vein (CV); C & D - Cisplatin injected group depicting damaged sinusoids (DS), inflammatory cells (IC), vacuolation (V) and bi-nucleated hepatocytes (BNH); E & F- Melatonin injected group showing liver histology similar to Control; G & H- Melatonin pre-treated followed by cisplatin injections denoting marked improvement in the histology of liver (CV, central vein).

(*p<0.01*) on the expression of Nrf-2 when compared with the cisplatin-only treated group. On the other hand, the expressions of HO-I, Cox-2, NF-κB and IL-1β all showed significant increase (*p<0.05, p<0.01*) following cisplatin treatment (Figure 9b – 9e). In the liver homogenate of animals which were pre-treated with melatonin to cisplatin injections, expressions of HO-I, Cox-2, NF-κB and IL-1β were decreased significantly (*p<0.01*) when compared with the cisplatin-only treated group.

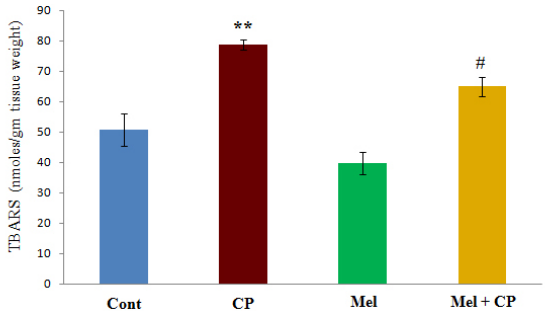


**Figure 6.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injections (15 mg/kg bw) on thiobarbituric acid reactive substances (TBARS) level in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

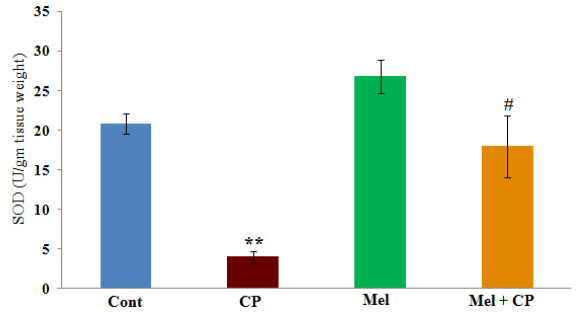


**Figure 7.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injections (15 mg/kg bw) on superoxide dismutase (SOD) activity in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

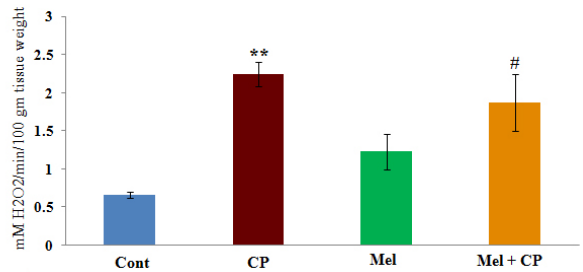


**Figure 8.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injections (15 mg/kg bw) on H2O2 depletion (catalase activity) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

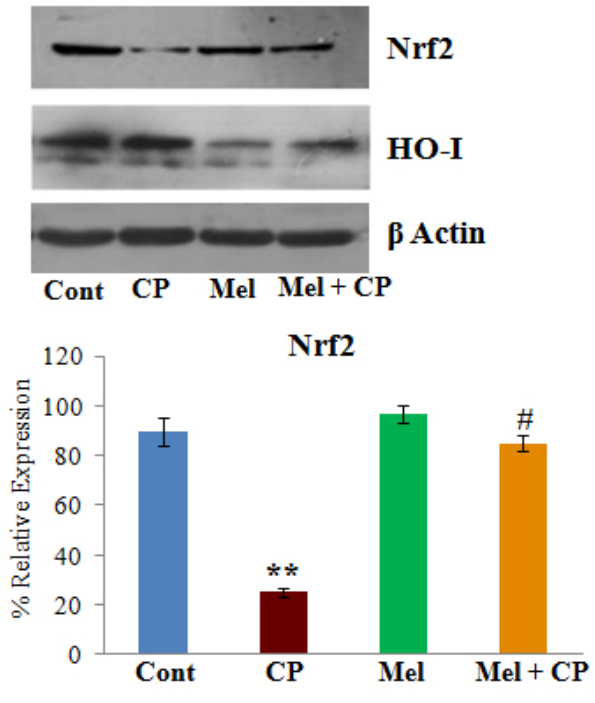


**Figure 9a.** Effect of melatonin pre-treatment (100 µg/100 g bw) and cisplatin injections (15 mg/kg bw) on the expression of nuclear factor erythroid 2–related factor 2 (Nrf2) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

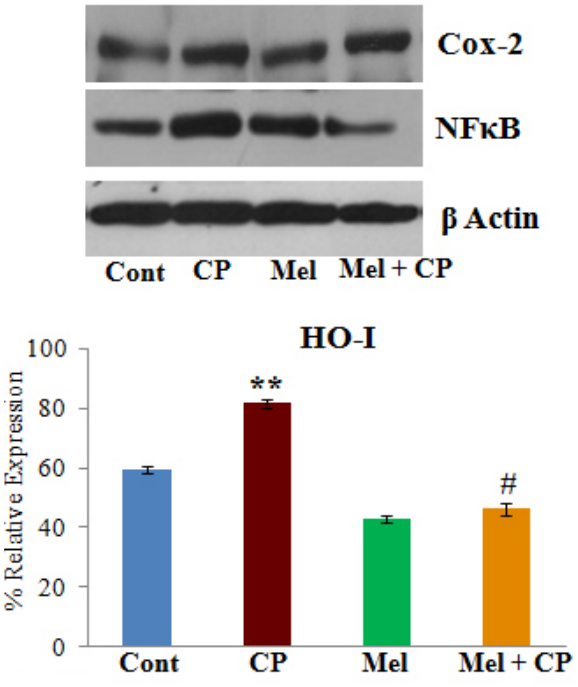


**Figure 9b.** Effect of melatonin pre-treatment (100 µg/100 g bw) and Cisplatin injections (15 mg/kg bw) on the expression of hemeoxygenase I (HO-I) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin.** p<0.01 Cont vs. CP. # p<0.01 CP vs.Mel+CP.

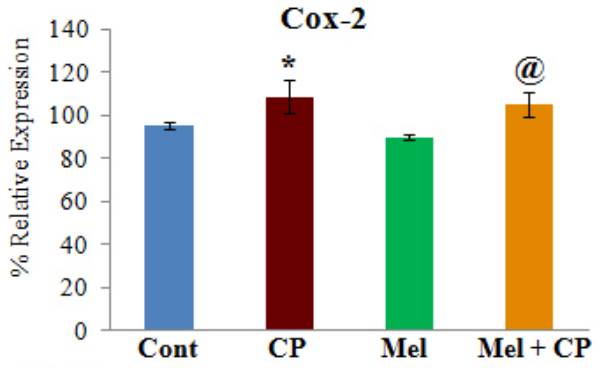


**Figure 9c.** Effect of melatonin pre-treatment (100 µg/100 g bw.) and cisplatin injections (15 mg/kg bw) on the expression of cycloxygenase-2 (Cox-2) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. * p<0.05 Cont vs. CP. @ p<0.05 CP vs.Mel+CP.

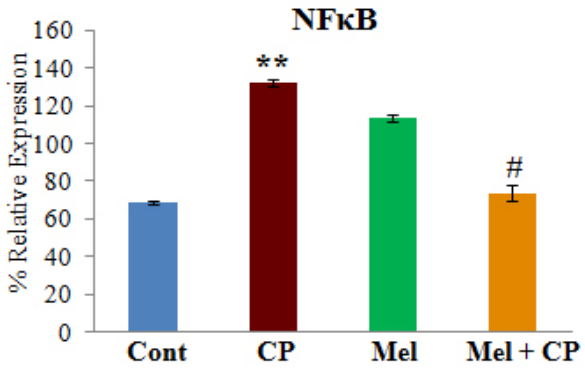


**Figure 9d.** Effect of melatonin pre-treatment (100 µg/100 g b. wt.) and Cisplatin injections (15 mg/kg bw) on the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by cisplatin. ** p<0.01 Cont vs. CP. @ p<0.05 CP vs.Mel+CP.

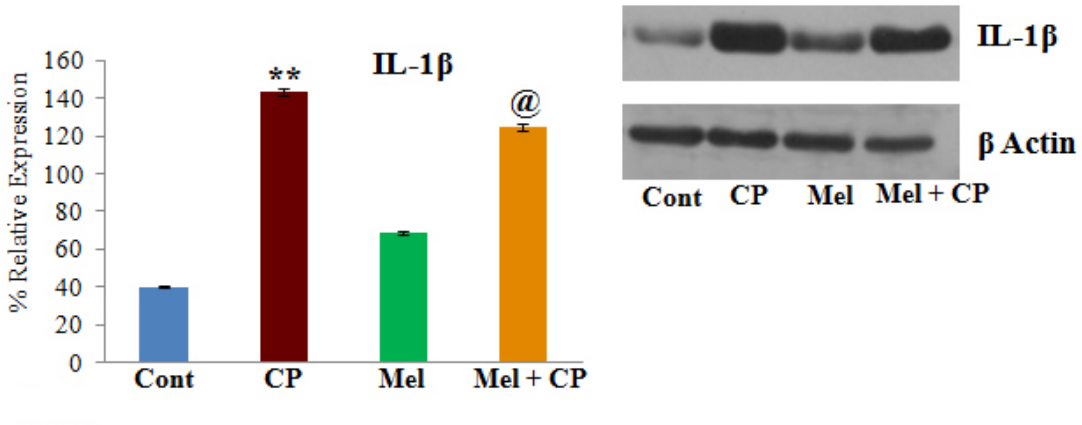


**Figure 9e.** Effect of melatonin pre-treatment (100 µg/100 g bw) and Cisplatin injections (15mg/kg b. wt.) on the expression of interleukin 1 beta (IL-1β) in the liver of male golden hamster *Mesocricetus auratus*. Vertical bars represent mean ± SEM, n= 5 for each group. Cont= Control; CP= Cisplatin injected; Mel= Melatonin-only injected; Mel+CP= Melatonin pre-treatment followed by Cisplatin. ** p<0.01 Cont vs. CP. @ p<0.05 CP vs.Mel+CP.

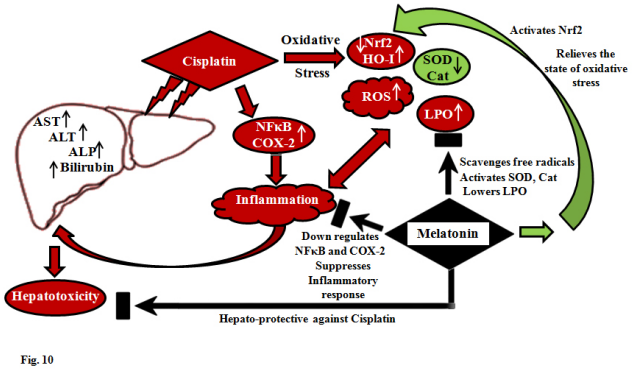


Fig. 10

**Figure 10.** Diagrammatic representation of the findings and conclusion of the study depicting the molecular mechanisms involved in cisplatin-mediated hepatic damage and the alleviation by melatonin.

## 4. Discussion

Our study shows that cisplatin treatment of golden hamster produces deleterious effects in the liver, which might be due to the generation and accumulation of ROS which in turn led to an inflammatory response as depicted by the levels of different pro-inflammatory molecules and resulted in failure of liver functions. Previous reports suggested renal toxicity of cisplatin[15,16] along with the some harmful effects on the liver[17]. However, the intricate molecular mechanism involved in the damage to the liver remained unanswered in those reports. The results of our study clearly suggest that cisplatin primarily alters the redox homeostasis of the liver and induces oxidative stress which in turn triggers inflammatory processes and finally results in loss of liver function.

The ability of cisplatin to alter liver function by increasing AST/ALT activities reported earlier in rats[18] is supported by our results in golden hamsters. Our histological analyses revealed the accumulation of inflammatory cells, necrotic hepatocytes and dilation of the central vein in cisplatin-treated animals. Hypertrophy and vacuolisation of the hepatocytes followed by karyorrhexis substantiate the nuclear damages and hence loss of function of the hepatocytes. All these alterations in the liver histology are identical to the changes observed in non-alcoholic fatty liver disease (NAFLD) which often leads to cirrhotic condition. The involvement of ROS in physiological pathologies is a very common event and in case of cisplatin-induced nephrotoxicity too[5]. The generation of ROS and decrease of the enzymatic antioxidants further promotes the process of renal failure[19]. The significant increase in the rate of lipid peroxidation measured by TBARS and the suppression of the activities of SOD and CAT measured in the liver support the involvement of oxidative stress induced by cisplatin. Further, the histological observations confirmed the steatohepatitis-like changes in the liver along with abnormally increased activities of AST, ALT and ALP proving the anomalies in liver function due to cisplatin treatment. Similar findings were reported in rats wherein cisplatin-induced liver failure was due

to increasing oxidative stress[20]. We therefore, extended our investigation to the molecular events that might be involved as downstream modulators of the whole process of liver damage induced by cisplatin.

ROS has been reported to trigger the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and up-regulate inflammatory responses[21,22]. NF-κB is an established oxidative stress-sensitive transcription factor and it regulates different genes required for maintenance of cellular homeostasis[23]. Therefore, NF-κB actively plays a positive role in promoting inflammation in cells under oxidative stress. Similarly, cyclooxygenase-2 (Cox-2) is an inducible form of cyclooxygenases and an important marker for inflammation[24]. Another critical cytokine-related to inflammatory responses is interleukin-1 beta (IL-1β) which is released by macrophages, and is known to activate Cox-2[25]. On the contrary, an important protein in the maintenance of cellular antioxidative defence is nuclear factor-like 2 (Nrf2) which regulates a wide variety of genes like that of the enzymatic antioxidants, Hemeoxygenase I (HO-I), etc[26]. HO-I is a stress-specific protein that gets activated upon induction of oxidative stress and catalyzes the breakdown of heme into biliverdin[27]. Nrf2-deficient mice have been reported to show increased NF-κB activation in response to lipopolysaccharide-mediated stress[28]. Hence, it can be suggested that pro-inflammatory markers and oxidative stress act together to exert the cellular damages that are involved in cisplatin-mediated liver failure.

Taking all the information into account we investigated the inflammatory processes generated, if any, as a possible conclusion for hepatic damages induced by cisplatin. The increased expression of NF-κB, IL-1β and Cox-2 strongly substantiated the induction of inflammation in the liver following cisplatin treatment. IL-1β is known to activate Cox-2[29] and we observed a similar condition, which affirms the involvement of IL-1β in triggering Cox-2 generation in hepatocytes following cisplatin treatment. The activation of HO-I supports the state of oxidative stress that the hepatocytes were subjected under the influence of cisplatin. Interestingly, the down-regulation of Nrf2 supported our hypothesis along with the results of the suppressed activities for SOD and Cat. Hence, we conclude that the hepatotoxicity induced by cisplatin-involved oxidative stress that further initiates inflammatory responses eventually culminating in failure of liver functions.

Since, oxidative stress is the primary initiator of the cellular damages, various studies have utilized different antioxidants such as epigallocatechin-3-gallate, chrysin, etc., to nullify the cisplatin mediated anomalies[15,30]. However, there have been only fewer studies involving melatonin as a possible antidote against cisplatin-mediated liver damages. Melatonin is an integral part of the body as it is synthesized primarily by the pineal gland. Reports have suggested that the efficacy of melatonin as an antioxidant surpasses that of most known antioxidants[31]. Melatonin is currently clinically used for sleep disruption (jet-lag) and shows no known side effects on the body physiology. Certain antioxidants at particular concentrations and the presence of metal ions such as $Fe^{2+}$ exhibit pro-oxidant properties by aiding in the process of the generation of $^{\cdot}OH$ radicals[31]. Such effects have never been reported for melatonin under any circulating or tissue concentrations. Therefore, we used melatonin as a preventive material to possibly neutralize the cisplatin-mediated liver damage. In the case of the radiation studies, it was well established that melatonin is effective only if administered prior to the incidence of radiation[14,32]. Therefore, we administered melatonin prior to cisplatin treatment.

Interestingly, our hypothesis is strongly supported by the results. Initial examination of liver histology showed significant improvement as discussed below in case of animals treated with melatonin prior to cisplatin injection. In the arrangement of hepatocytes, sinusoids were almost similar as in normal liver histology. The decrease in the activity of AST, ALT and ALP are directed towards a normal functioning of the liver. The free radical scavenging property of melatonin suppressed the state of oxidative stress that was prevalent in cisplatin-treated ones. Lowered lipid peroxidation and activated enzymatic antioxidants proved the prevention of the oxidative stress that was otherwise induced following cisplatin treatment. The results of the expression patterns of the pro-inflammatory markers also showed marked improvement in the melatonin-treated animals. Melatonin is known to down-regulate NF-κB and Cox-2[33,34]. In our results the expressions of these pro-inflammatory markers were also down-regulated depicting the anti-inflammatory property of melatonin. The down-regulation of IL-1β in the melatonin-treated animals might also have helped in the deactivation of Cox-2. The increase in Nrf2 expression substantiates the antioxidative role of melatonin as it is reported to activate Nrf2 as a response against oxidative stress[16]. The oxidative stress maker protein HO-I was also

suppressed in the melatonin-treated group indicating the maintenance of the redox homeostasis of the cells and suppression of oxidative stress. Hence, the pre-treatment of melatonin neutralized the hepatotoxicity induced by cisplatin. Therefore, it can be hypothesized that a pre-treatment of melatonin will definitely be a promising support for chemotherapy since melatonin is well-tolerated in the body and has low interaction with other medications[35].

## 5. Conclusion

Our results strongly support the hypothesis that liver damage incurred due to cisplatin treatment can be ameliorated by melatonin pre-treatment. The oxidative stress-mediated inflammatory response as reflected by expressions of NF-κB, Cox-2 and IL-1β in the liver following cisplatin treatment proved to be the main cause of liver failure. The administration of melatonin prior to cisplatin helped in neutralization of ROS and suppression of pro-inflammatory molecules owing to its anti-inflammatory properties. Therefore, it can be proposed that administration of melatonin followed by cisplatin might be helpful as a therapeutic agent for cancer treatment as this nullifies the ROS and inflammation caused by cisplatin while keeping its anti-cancer properties intact. Although too early to prescribe such a formulation, this could yet be a new approach of utilizing the anti-neoplastic attributes of cisplatin which is generally compromised due to the harmful side-effects.

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## 7. Conflict of Interest

The authors report no conflicts of interest either personal or financial.

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