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" *Melatonin alleviates cisplatin induced liver toxicity in rats by up-regulation of Nrf2/HO-1 signaling pathway* "

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ABSTRACT:

Background: One of the most well-known chemotherapy drugs is cisplatin (Cis). Hepatotoxicity is among the most significant adverse effects of cisplatin. The pineal gland hormone melatonin (Mel) is primarily reliant on the light/dark cycle for its cyclic release. This neurohormone can preserve tissue redox equilibrium and functions as an antioxidant.

Aim: The purpose of the study was to assess hepatoprotective impact of Mel in rats exposed to Cis, as well as any possible underlying processes.

Methods: Three groups of thirty adult male albino rats were created: control, Cis, and Cis+Mel. Hepatic MDA, hepatic SOD, hepatic TNF-α, hepatic IL-6, hepatic IL-10, liver-expressed Nrf2 and HO-1 genes and serum liver enzymes were measured. Additional assessments of the liver's caspase-3 and NF-kB immunoreaction were conducted.

Results: Hepatic SOD, IL-10, and Nrf2 and HO-1 gene expression all significantly decline in response to cis-induced damage. Nevertheless, as compared to control, there was a notable rise in serum liver enzymes along with hepatic MDA, TNF-α, and IL-6, along with an upregulation of caspase-3 and NF-kB immunoreaction in the liver. Mel significantly improved the liver abnormalities brought on by Cis.

Conclusion: Mel employed anti-oxidant, anti-inflammatory, and anti-apoptotic mechanisms in addition to up-regulating the Nrf2/HO-1 signaling cascade to reduce the hepatotoxicity brought on by Cis.

Key words: Caspase-3, Cisplatin, Hepatotoxicity, HO-1, Melatonin, NF-kB, Nrf2,

Introduction

A significant amount of the world's illness burden is caused by cancer, and forecasts indicate that the prevalence of cancer will continue to climb globally for at least the next 20 years. Even while advancements have led to improved cancer detection and treatment techniques, higher survival rates are often accompanied by treatment-related toxicities. Cisplatin (Cis) is a powerful chemotherapy medication that is widely used to

treat a variety of cancers. Despite its efficacy, Cis's clinical use is restricted because of its side effects, especially hepatotoxicity, which frequently calls for dosage changes or therapy termination, jeopardizing therapeutic results (Styam et al., 2024).
Cis uses oxidative stress and DNA crosslinking to halt mitosis and trigger apoptosis. The main mechanisms by which cis kills cancer cells are believed to be DNA adduct formation, G2 cell cycle arrest, and apoptosis (Mir et al., 2015). Hepatotoxicity and nephrotoxicity are linked to cisplatin treatment since it is mostly metabolized by the kidney and liver (Mir et al., 2015).

Cis-induced hepatotoxicity mechanism reports that the drug may disrupt the tissue antioxidants and produce excessively ROS. As a result, Cis may harm the liver oxidatively (Ezz-Din et al., 2011).
When Cis builds up inside the liver cells, it can cause hepatotoxicity. An increase in OS, inflammation, and apoptosis activation are the main mechanisms behind Cis's hepatotoxicity (El-Sharouny et al., 2019). Cell penetration by Cis occurs by passive transport. The cytochrome P450, CYP450 enzyme complex exposes Cis to hepatic metabolism and biotransformation upon its entry into the cells (Lu et al., 2018)..

Furthermore, individuals who received low doses of Cis have experienced hepatotoxicity, most likely due to the cumulative effects that culminate in substantial hepatic toxicity, including necrosis, inflammatory lesions, and the disintegration of hepatic cords (Singh et al., 2015).

The mechanism of Cis hepatotoxicity may entail proinflammatory gene activation, apoptosis, disruption of Ca2+ homeostasis, and structural and functional mitochondrial damage (Mir et al., 2015).
Nrf2 is an essential transcription factor that regulates responses to oxidative stress. Nrf2 activation can then boost the expression of antioxidant factors like HO-1 to lessen oxidative stress harm. (Krisnamurti et al., 2022). In order to protect the liver from oxidative damage, Nrf2 activation was essential. Furthermore, it has been demonstrated that activation of the Nrf2 pathway lowers proinflammatory response and oxidative stress. (Zeng et al., 2021).

One of the main mediators for regulating redox hemostasis is the Nrf2/HO-1 signaling pathway. When cellular redox homeostasis is disturbed, the amount of inflammatory

mediators, such NF-κB, which are essential for hepatocyte regeneration and repair, fluctuates. Therefore, it is necessary to activate Nrf2/HO-1 to restore redox balance. (Sedik & Amer, 2022)

The expression of HO-1 is controlled by Nrf2. Additionally, it has been shown that Nrf2 alters the transcription of multiple genes that regulate the cell's anti-inflammatory and antioxidant pathways under normal conditions (Awad et l., 2023).

The pineal gland hormone melatonin (Mel) is primarily reliant on the light/dark cycle for its cyclic release. Its physiological effects also depend on this neurohormone's ability to preserve tissue redox equilibrium and act as an antioxidant (Ristić et al., 2020).

This study also uses Mel, that has antioxidant and anti-inflammatory properties, as a potential hepatoprotective medication. Additionally, melatonin has anti-cancer properties. Additionally, it has no negative side effects, even at high dosages. Mel lessens the negative effects of chemotherapy, including the immune response (Mansour et al., 2024).

Furthermore, if cis and Mel are taken in combination therapy, there is no functional interaction between the two medications, Mel's anti-cancer properties may be an additional advantage (Goswami et al., 2021).

Numerous processes, including as electron and/or hydrogen transfer, the creation of radical adducts, metal chelation, and the restoration of biological targets, are linked to Mel's antioxidant action (Ristić et al., 2020)

With reference to the Nrf2/HO-1 signaling system, our goal in this study was to elucidate the hepato-protective impact of Mel in liver damage caused by Cis and the potential underlying processes.

Materials and Methods

Animals

This study was carried out in accordance with the rules established by the Institutional Animal Care and Use Committee (IACUC), Menoufia University, with IRB No: MUFS/F/GE/3/25. Thirty mature male Wister rats, weighing 150–180 g, were used. Regular rat food and tap water were also freely available to them.

Experimental design

Rats were randomly divided into (10 rats each):

Control group: For twenty-one days, each rat received an i.p. injection of an equivalent volume of Mel vehicle, which was used to dissolve the medicines. On the sixteenth day of the experiment, each rat received a single i.p. injection of 1 ml of normal saline.

Cisplatin group: The animals received a single dose of Cis (10 mg/kg body weight i.p) on the 16th day of experiment according to (Yahya et al., 2023), Cis (CAS No. 15663-27-1) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). For 21 days starting from the 1st day of study, the rat was also given an intraperitoneal injection of an equivalent volume of the Mel vehicle, which was used to dissolve the medicines.

Cisplatin /Melatonin group: On the sixteenth day of the experiment, the rats were given a single dosage of Cis (7 mg/kg body weight i.p.) along with melatonin (4 mg/kg body weight i.p. for 21 days starting from the 1st day of study), (Mansour et al., 2024), which was acquired from Bio Basic Inc., Canada.

Melatonin preparation

To achieve a concentration of 0.1 mg/ml, 100 mg of melatonin powder was dissolved in 1 ml of 100% ethanol and combined with 1 liter of tap water. The melatonin solution was made from scratch. Dark foil was used to conceal the melatonin solution vials (Shen et al., 2002).

At the end of the 21-day study, all of the rats were sacrificed, and the liver was immediately dissected after being weighed. The liver's right lobe was maintained at -80°C for the Nrf2 and HO-1 genes' real-time PCR investigation. The remaining liver lobe sections part were homogenized for biochemical analysis, while the remaining liver sections were produced for immunohistochemical analyses.

Blood sampling and biochemical analysis

Following an overnight fast, samples of retro-orbital blood were drawn, centrifuged for 15 minutes at 2000 rpm, and allowed to coagulate for 30 minutes at room temperature. Serum was obtained, frozen at -80°C, and its liver enzymes ALT, AST, ALP, and GGT

were examined using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt).

Tissue homogenate preparation

A tissue homogenizer was used to independently homogenize liver tissues. The crude tissue homogenate was centrifuged in an ice-cold centrifuge for 13 minutes at 13,000 rpm. Hepatic MDA and SOD levels were assessed using colorimetric kits (Biodiagnostic Company, Dokki, Giza, Egypt).

Following the manufacturer's instructions, the levels of hepatic IL-10, IL-6, and TNF-α were measured using ELISA (IL-10: ERI3010-1, Assaypro LLC, Saint Charles, Missouri, USA; TNF-α: ERT2010-1, Assaypro LLC, Saint Charles, Missouri, USA; and IL-6: ab100772, Abcam, Cambridge, UK).

Quantitative assay of gene expressionusing RT-PCR

The relative mRNA levels of the liver's Nrf2 and HO-1 genes were evaluated using RT-PCR. Total RNA was extracted from tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA was stored at -80°C until it was required. In the first PCR phase, cDNA synthesis (reverse transcription step) was carried out using ThermoScriptTM RT reagent kits (Invitrogen). After that, cDNAs were amplified using SYBR Green Mix kits (Stratagene, USA) in PCR experiments. From each amplification curve, a cycle threshold (Ct) value was derived. As the reference gene, GAPDH was employed. 7500 ABI PRISM (Applied Biosystems, USA) v.2.0.1 was used for data analysis. The comparative □□Ct technique was utilized to quantify the relative quantification of Nrf2 and HO-1 gene expression. The Nrf2 and HO-1 gene primers used were as follows:

The following primers were used for the Nrf2 gene:
(1) Forward primer: 5- GGTTGCCCACATTCCCAAATC-3
(2) Reverse primer: 5- CAAGTGACTGAAACGTAGCCG-3
The following primers were used for the HO-1 gene:
(1) Forward primer: 5-AGGTGCACATCCGTGCAGAG-3
(2) Reverse primer: 5-CTTCCAGGGCCGTATAGATATGGTA-3 (Fouad et al., 2025).

Histopathological analysis

Fresh liver samples were taken from the right lobe and immediately stored in neutral buffered formalin (10%). H & E were used to stain paraffin slices, which were 5 µm

thick, in order to verify histological features.

Rat monoclonal primary antibody against NF-kB (monoclonal, dilution 1:200, Abcam) and caspase-3 (rabbit polyclonal antibody, Dako, Carpinteria, California, USA) was the primary monoclonal antibody utilized. The cytoplasm of the cells appeared brownish, indicating the cellular reaction. The manufacturer's instructions for the immunostaining process were followed.

Statistical analysis

The data was analyzed using SPSS software, version 16 (SPSS, Inc., USA). The statistical data was displayed as mean±SD,. After determining the significance of group differences using one-way analysis of variance, a post hoc Tukey test was conducted. A p-value of less than 0.05 was considered statistically significant.

Results

Serum liver enzymes, hepatic MDA, TNF-α, and IL-6 were substantially raised in the Cis compared to the control, while hepatic SOD, hepatic IL-10, and hepatic Nrf2 and HO-1 gene expression were substantially reduced. In comparison to the Cis, Cis+Mel demonstrated significantly higher expression of the hepatic Nrf2 and HO-1 genes, hepatic SOD, heptic IL-10, significantly lower serum liver enzymes, hepatic MDA, TNF-α, and IL-6, as well.

Table (1): The measured serum liver enzymes, hepatic MDA, SOD, TNF-α, IL-6, IL-10 , hepatic Nrf2 and HO-1 genes expression in all studied groups

	Control group	**Cis group**	**Cis + Mel group**
Serum ALT (U/L)	42.9±4.5	168.99±5.8 *	**99.8±3.1** *#
Serum AST (U/L)	51.9±3.2	211±6.5 *	**129.8±4.88** *#
ALP (U/L) Serum	91.8±4.3	260.9±2.99 *	**163.9±5.5** *#
GGT (U/L) Serum	4.7±0.11	18.3±1.03 *	**10.99±1.09** *#
Hepatic MDA (nmol/ gm. Tissue)	6.32 ±0.8	22.9± 1.9*	**12.9± 0.97***#
Hepatic SOD (U/gm. Tissue)	8.64±0.22	2.01±0.33*	**6.18±0.77***#
Hepatic TNF-α (ng/ml)	26.8±3.2	55.8±3.1*	**38.9±3.2***#
Hepatic IL-6 (pg/mL)	70.8±4.1	193.9±3.65*	**129.8±2.9***#
Hepatic IL-10 (ng/mL)	22.5±2.01	6.99±0.81*	**14.8±1.01***#
Nrf2 gene expression	1	0.36±0.07*	**0.68±0.06***#
Hepatic HO-1 gene expression	1	0.41±0.09*	**0.73±0.04***#

* Significant compared with control, # Significant compared with Cis.

Hematoxylin and Eosin staining:

The hepatic parenchyma displayed intact hepatocytes with central vesicular nuclei, while sections from the control displayed normal histological liver structure with a normal central vein (Fig. 1A). Severe central vein dilatation and congestion, along with severe perivascular inflammatory cell infiltrations, were observed in the Cis group (Fig. 1B). Although the hepatocytes appeared to be normal based on the Cis+Mel, modest inflammatory cell infiltrations were still visible (Fig. 1C).

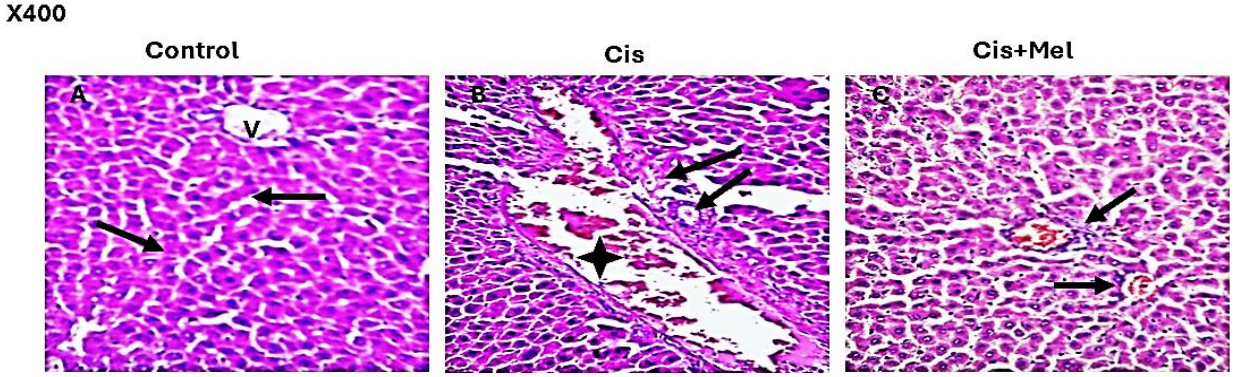

Fig. 1: (H&E ×400): A photomicrograph of the control group in (A) revealed intact

hepatocytes with central vesicular nuclei (arrows) and a normal histological appearance of the liver structure with a normal central vein (V). (B) The Cis group displayed severe perivascular inflammatory cell infiltrates (arrows) together with severe dilatation and congestion in the central vein (star). (C) The Cis+Mel indicated that although the hepatocytes appeared to be normal, there were still some minor inflammatory cell infiltrations visible (arrows).

Immunohistochemical results

In the Caspase-3 stain, the Cis group's percentage area of Caspase-3 was substantially higher than control (80.5±0.02 vs. 9.4±0.05, $p<0.05$). This proportion was higher than that of the control, but it was dramatically lower in the Cis+Mel than in the Cis group (23.2±0.13 vs. 80.5±0.02, $p<0.05$). (Fig. 2: A-D).

In the NF-kB stain, the Cis group's percentage area of NF-kB was substantially higher than control (83.7±0.05 vs. 6.2±0.03, $p<0.05$). This proportion was higher than that of the control, but it substantially decreased in the Cis+Mel when compared to the Cis (33.5±0.12 vs. 83.7±0.05, $p<0.05$). (Fig. 2: A-D).

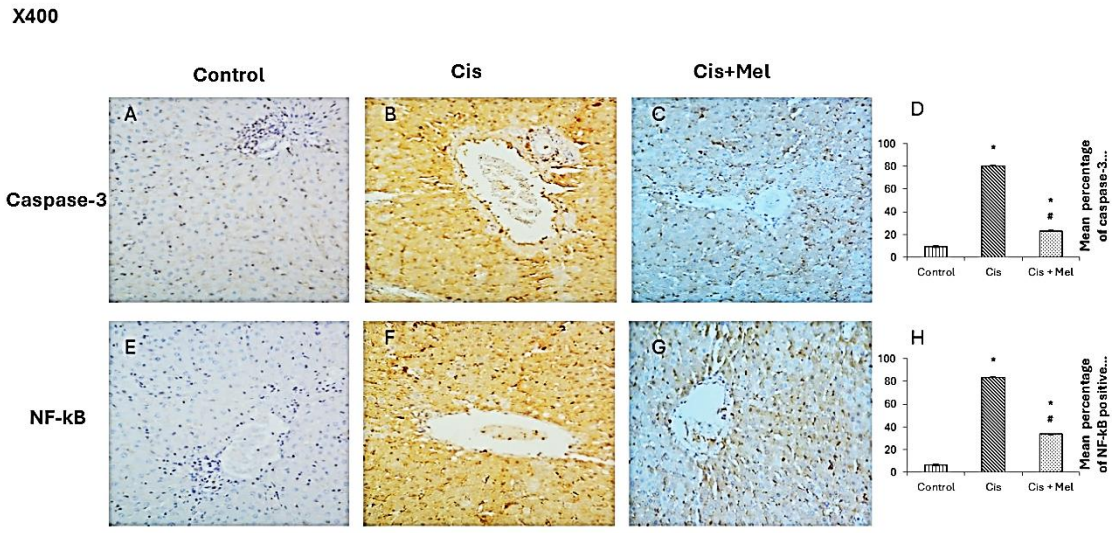

Fig (2): The Caspase-3 (A-D) and NF-kB (E-H) immunoreactions significantly increased in the Cis group and substantially decreased in the Cis+ Mel, according to representative micrographs of the various experimental groups. Magnification of x400.

Discussion

A tiny chemical that readily penetrates cell membranes to reach the nucleus and alter DNA structure, Cis is an anti-cancer medication used to treat solid organ cancers. The pineal gland is the primary source of the hormone melatonin. Melatonin's effectiveness as a potent antioxidant has been shown in numerous research (Mansour et al., 2024).

In the current investigation, we employed melatonin as a preventative measure to lessen the liver damage brought on by Cis. The WHO-recommended most sensitive parameters for the liver cell function test. The two main indicators used to gauge the degree of liver cell damage are ALT and AST (Mir, et al., 2015).

The current study's biochemical findings demonstrated that Cis caused hepatotoxicity following a single dosage. Our histological results revealed that the liver enzyme blood levels in the Cis group were considerably higher than those in the control, indicating hepatocellular damage in agreement with (Louisa et al., 2023).

When Cis builds up inside the liver cells, it can cause hepatotoxicity. The main processes that underlie Cis's hepatotoxicity include an increase in oxidative stress, inflammation, and apoptosis induction (Mansour et al., 2024).

This increase in hepatic enzymes is considerably reduced by co-administration of melatonin. This result was consistent with prior research that linked melatonin's hepatoprotective effects to its anti-inflammatory and antioxidant impacts (Mansour et al., 2024).

Oxidative stress and apoptosis activation are the primary mechanisms of cis-induced hepatotoxicity (Taghizadeh et al., 2021). ROS overproduction and the depletion of antioxidant system lead to OS. The liver cells' membrane lipids and other biological components were harmed by oxidative stress. Heptotoxicity and its histological modification were linked to Cis administration since the liver also has a propensity to store a sizable amount of the drug (Bona et al., 2018).

This was demonstrated in our study by significantly higher MDA and lower SOD in the Cis group when compared to the control, which is consistent with other research (Ko et al., 2014, Louisa et al., 2023).

Mel, however, significantly reduced the oxidative stress brought on by Cis, as seen by lower MDA and higher SOD levels in the Cis+Mel group as opposed to the Cis group, which is consistent with earlier research (Ristić et al., 2020).

Mel's capacity to either scavenge the generated oxygen radicals or suppress the activity of xanthine oxidase is what gives it its antioxidant properties (Ristić et al., 2020). Additionally, Mel directly scavenges free radicals by regulating the genes of antioxidant enzymes. Its ability to protect macromolecules, from oxidative damage through oxidant scavenging properties is becoming more and more clear. Furthermore, because it activates antioxidant enzymes like glutathione and superoxide dismutase, its antioxidant qualities may be explained (Khodir et al., 2024).

Cis-induced hepatotoxicity is significantly influenced by inflammation. The transcriptional activation of TNF-α and IL-6 can be facilitated by NF-κB (Fu et al., 2023).

In line with earlier research, our data demonstrated that exposure to Cis significantly increased hepatic TNF-α and IL-6, decreased anti-inflammatory IL-10, and up-regulated hepatic NF-kB immunoreaction in Cis compared to control (Zhang et al., 2024). Furthermore, Wei et al. showed that Nrf2 can partially block NF-κB activation through its downstream gene, HO-1 (Gao et al., 2021).

This finding is consistent with a prior study that demonstrated Cis's toxic effects on the liver, which raise inflammatory cytokine levels in the liver via the extrinsic pathway, in which the ligand attaches to TNF-α, recruits procaspase-8, and creates a signaling complex that causes death. (Dasari and Tchounwou, 2014).

However, Mel significantly reduced the inflammatory condition in the liver caused by Cis, which is consistent with other research (Kaymak et al., 2022). Mel has been demonstrated to dramatically lower TNF-α levels in liver damage caused by carbon tetrachloride (Ebaid et al., 2013)

Additionally, compared to the Cis group, melatonin reduced the expressions of hepatic NF-κB immunoreaction, indicating the anti-inflammatory effect of Mel and supporting earlier research (Lim, et al., 2012).

A downstream regulator of the HO-1 protein, Nrf2 is an antioxidant transcription factor that coordinates antioxidant signals necessary for maintaining cellular redox homeostasis. Numerous investigations have demonstrated that the Nrf2/HO-1 pathway

has strong ROS scavenging capabilities and, through its anti-inflammatory and antioxidant qualities, contributes to liver cytoprotection. Nrf2 suppresses NF-kB and pro-inflammatory mediators. (Okkay et al.,2024)

Therefore, the SOD in this study results from the mechanistic depression of the antioxidant and anti-inflammatory protein, HO-1, which is promoted by the reduction in Nrf2/HO-1 hepatic gene expression caused by Cis toxicity. Our experimental results showed that Nrf2 and HO-1 mRNA expression were significantly reduced in the livers of Cis rats. Additionally, this is consistent with earlier studies (Zhang et al., 2024, Louisa et al., 2023)

Nonetheless, Mel increased the expression of the hepatic Nrf2/HO-1 gene in the Cis+Mel relative to the Cis, which is consistent with other research (Kilic et al., 2013, De Araujo et al., 2019). Melatonin activates Nrf2, which raises the mRNA and protein levels of antioxidant enzymes. Mel's upregulation of Nrf2 led to a rise in the expression of the antioxidant enzyme HO-1 (Kilic et al., 2013).

Caspases are a family of protease enzymes necessary for programmed cell death, and proteolytic caspases have a role in the initiation and completion of apoptosis in inflammation (Fouad et al., 2025).According to the data, the Cis group had a higher Caspase-3 hepatic immunoreaction than the control group, which is consistent with earlier research (Louisa et al., 2023).

The hepatotoxicity from cisplatin administration, demonstrated by elevated AST and ALT levels, which raise hepatocyte cell death, can account for the rising expressions of caspases' mRNA in the Cis group. (Omar et al., 2016).

In contrast to the Cis group, Mel significantly reduced the hepatic caspase-3 immunoreaction. This is consistent with a prior study that found that Mel inhibited an increase in caspase-3 activity, which may have been caused by its capacity to regulate cytochrome C release or by blocking the lipid peroxidation process (Ristić et al., 2020).

Conclusion

Mel employed anti-oxidant, anti-inflammatory, and anti-apoptotic mechanisms in addition to up-regulating the Nrf2/HO-1 signaling cascade to reduce the hepatotoxicity brought on by Cis.

Conflict of interestAuthors declare that there is no conflict of interest

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