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Research Article

Protective effect of melatonin on cisplatin induced liver toxicity inalbino Rats; biochemical and histological Study

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Abstract

Background: Cisplatin is a potent chemotherapeutic agent, widely used in treatment of cancer. Melatonin, body hormone secreted by the pineal gland, has anti inflammatory and antioxidant characters. This study was done to evaluate the protective role of melatonin on cisplatin-induced hepatotoxicity in rats. **Materials and Methods**: Forty rats were divided into four groups: group I(control group) in which, the animals received no drug), group II (melatonin group) receiving melatonin (4mg/kg i.p. for 10 days), group III (cisplatin group) receiving (single dose of 7 mg/kg), and group IV (melatonin/cisplatin group) receiving melatonin for 10 days+ cisplatin with a single dose on the 5th day of experiment. Drugs were given by intraperitoneal injection. At the end of experiment, blood samples were obtained from the tails of animals for biochemical study then all rats were sacrificed and livers were dissected out for histological study. **Results**: Cisplatin increased liver enzymes (ALT, AST). Cisplatin induced histopathological changes such as vaculation of hepatocytes, sinusoidal dilatation, congestion of central and portal veins, and inflammatory cell infiltration. Cisplatin significantly decreases PAS reaction in hepatocytes and increases collagen fiber deposition. Melatonin administration significantly ameliorated these changes. **Conclusion:** melatonin with its antioxidant and anti-inflammatory properties can be recommended as a promising medication in treatment of cisplatin induced hepatotoxicity in cancer patients.

Key words: melatonin, cisplatin, liver toxicity

Introduction

It has been demonstrated that the inorganic platinum-based drug, cisplatin is extremely effective in the treatment of cancer, one of the most common and deadly illnesses of this century. Cisplatin is used for the treatment of various types of cancers including lung, breast, ovarian, testicular and bladder. Cisplatin causes cross linking with purine bases of DNA in the cancer cells, stopping its replication, thus inhibiting the growth of these tumor cells[1,2]. It makes this important effect at the expense of damaging other organs with dangerous adverse-effects as neurotoxicity, nephrotoxicity, gastro-intestinal toxicity and hepatotoxicity[3]. Oxidative stress is the imbalance between free radicals and natural antioxidants in the body. While oxidative stress caused by cisplatin is helpful in death of cancer cells, it also leaves healthy tissues vulnerable to be damaged from reactive oxygen species (ROS)[4].

The primary organ for the metabolism and detoxification of endobiotics and xenobiotics is the liver[5]. Hepatotoxicity may result from the accumulation of cisplatin within the liver cells. The primary mechanisms underlying the hepatotoxicity by cisplatin include an increase in oxidative stress, inflammation, and induction of apoptosis[6]. Through passive transport, cisplatin penetrates the cell. When cisplatin enters the cells, it is exposed to hepatic

metabolism and biotransformation via the cytochrome P450, CYP450 enzyme complex[7]. The main enzyme implicated in hepatotoxicity according to the literature is a cytochrome P450 2E1 (CYP2E1) enzyme[8]. Additionally, hepatotoxicity has been seen in patients who received low doses of cisplatin, most likely as a result of the cumulative effects, which causes significant hepatic toxicity, including the disintegration of hepatic cords, inflammatory lesions and necrosis. [9].

Melatonin, a hormone with anti-inflammatory and antioxidant characteristics, is also used in this study as a possible hepatoprotective drug. Melatonin also posses anti-cancer property [10]. Also it has no harmful adverse effects even with high concentrations [11]. Melatonin reduces chemotherapeutic adverse effects such immunological response and thrombocytopenia. [12]. Additionaly, if melatonin is used in combination therapy with cisplatin, the two drugs don't functionally interact with one another, the anti-cancer action of melatonin may be an added benefit[10].

Materials and Methods

Animals:

The study used forty adult albino rats (average weight: 180-200 g). These rats came from Minia University animal house. They received water and a typical laboratory diet while being housed during the course of the experiment. All rats were kept in an air-conditioned, well-ventilated environment that was kept at aconstant 22°C. Every aspect of animal care and handling adhered to the ethical standardsset forth by the College of Medicine at Minia University in Egypt. Approval number 701:12/2020 in compliance with world standards.

Experimental work:

Our work was prepared in the Anatomy Department, Faculty of Medicine, Minia University, Egypt. The animals were divided into four groups ten rats for each.

- **Group I (control group):** The animals received adequate amounts of food and water.
- **Group II (melatonin group):** The animals injected with melatonin(4mg/kg body weight i.pfor10 days) according to[13].
- **Group III (cisplatin group):** The animals received a single dose of cisplatin (7mg/kg bodyweight i.p) according to[14].
- **Group IV (cisplatin/melatonin group):** The animals received melatonin (4mg/kg body weight i.p for10 days) + single dose of cisplatin (7mg/kg body weight i.p) on the fifth day of the experiment.

By the end of experiment, blood samples were taken from the tail vein and put into tubes with EDTA for biochemical study then, animals were sacrificed and liver was removed and prepared for microscopic examination.

Biochemical study:

The blood was centrifuged at 3,000 rpm (round per minute) for 10 minutes, after staying for 15 minutes at room temperature. The biochemical parameters, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were calculated in the serum using (Reactive GPL, Barcelona, Espana) commercial kits [15].

Histological studies by light microscope:

a- Hematoxylin and eosin stain (H&E) according to[16]

Following normal procedures, the liver was removed and fixed in 10% buffered formalin for 2 days before being embedded in paraffin**.** Dehydration with series grades of ethyl alcohol, embedded in 70% alcohol for 24hours, then in 90% alcohol for two hours, and then in 100% alcohol for half of hour, clearing with xylene, three successive changes of impregnation at 55°–60°C in soft paraffin, and finally embedding in hard paraffin wax after fixation of the liver tissue. A rotatory microtome was used to cut serial transverse sections that were 5µm thick, which were subsequently, put on glass slides. After being deparaffinized and stained with hematoxylin and eosin, the paraffin slices were rinsed with water. The sections were cleaned in xylene and dehydrated in alcohol before mounting.

b- Periodic acid-Schiff (PAS) technique: According to [16]

The PAS technique involves subjecting the tissue to periodic acid. This functions as an oxidizing agent that oxidizes glycol groups or derivatives of amino/alkyl aminos. Dialdehydes

are created by this oxidation. When subjected to Schiff's reagent, these dialdehydes produce an insoluble magenta product. Sections of 3µm thicknesses were deparaffinized with xylene and stained with periodic acid for10-15. The sections were washed for 5-10 minutes by running tap water followed by exposing to Schiff's reagent for10-15minutes. The sections were washed in running tap water for5-10 minutes, dehydrated in ascending concentrations of alcohol and then cleared by using xylol.

Result, the positive sites appeared magneta red staining glycogen granules.

c- Mallory's trichrome stain:

According to[17], It is appropriate to use neutral buffered, formalin-fixed tissue in 5 m paraffin sections.

Sections fixed in Bouin's solution as a secondary fixation may be beneficial for tissues fixed in formalin.

Results**;** nuclei appeared red, erythrocytes appeared orange, muscle appeared red and collagen appeared blue

Morphometric studies

Form each group ten random fields/section from each animal were chosen. The percentage of surface area fraction of PAS[18] and Mallory's trichrome[19]stained sections of liver tissue of all groups were recorded using software image analysis image J program[20]. The mean surface area fraction was used to compare between the different groups.

Statistical analysis:

Analysis of the data was done using the statistical package software, IBM SPSS 28.0 (IBM; Armonk, NewYork, USA). Ordinary one-way ANOVA test for non-parametric quantitative data between the four group [21]. The statistically significant differences between groups were determined using an unpaired t-test between every two groups. Statistics were considered significant at p 0.05[22]**.**

Image capturing:

A high-resolution colour digital camera placed on an Olympus microscope was used to take pictures of fields. These pictures were then uploaded to a computer to be analyzed.

Results

1- Biochemical results:

The serum level of hepatospecific markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in blood samples of the different groups. The results revealed that cisplatin injection significantly increased serum ALT and AST levels as compared to the control group. Concomitant treatment of melatonin and cisplatin caused significant decrease in ALT and AST values as shown in (table.1).

2- Histological results:

a) Hematoxylin& Eosin-stained sections results

The liver sections of both **control and melatonin** groups presented a normal lobular architecture. The hepatocytes radiated from the central vein, with hepatic sinusoids in between. The hepatocytes are polygonal with large rounded nuclei and acidophilic cytoplasm. Their nuclei were vesicular with prominent nucleoli (Fig.1, 2). In the **cisplatin group,** Liver sections showed distorted hepatic architecture and marked dilated sinusoids particularly in the central regions of the hepatic acini with marked dilatation of the central vein and portal vein. There is marked vaculation of hepatocytes especially around the central vein. Inflammatory cell infiltration appeared in pericentral area (Fig.4). Hypereosinophilic cytoplasm and condensed, darkly stained nuclei, often known as pyknotic nuclei, are characteristics of apoptotic hepatocytes. (Fig.4).

In the cisplatin - melatonin treated group, there is marked reduction of these changes had observed in the liver sections of this group. There was nearly restoration of lobular architecture with less dilated central vein and mildly dilated blood sinusoids (Fig.5,6).

b) Periodic Acid Schiff reaction (PAS)

Liver sections of the **control and melatonin** groups showed that the cytoplasm of hepatocytes was PAS-positive for glycogen granules, indicating that the hepatocyts were normal and had high glycogen content (Fig.7,8). In **Cisplatin group**, liver sections of the cisplatin group demonstrated a reduction in the glycogen content of the hepatocytes, as evidenced by the weak PAS reaction in certain cells and the absence of the reaction in other cells (Fig.9). In **Cisplatin-melatonin** group**,** liver sections of the cisplatin-melatonin group

revealed relatively stronger PAS reaction in hepatocytes as compared with the cisplatin group (Fig.10).

c) Mallory's trichrome stain

Mallory-stained sections of both **control and melatonin** groups showed minimal collagen fibers around the central veins, and in the region of the portal tract (Fig.11, 12). In Cisplatin group, Mallory-stained sections of the cisplatin group revealed excess collagen fiber deposition around the central vein (Fig.13). In **Cisplatin-melatonin** group**,** sections of the cisplatin-melatonin group revealed little collagen fiber deposition around the portal tract compared to the cisplatin group (Fig.14).

3- Morphometric results

A) Surface area fraction of PAS-positive stained liver tissues

There is a normal percentage in control and melatonin groups but highly decreased in the cisplatin group while it restored its elevation in the cisplatin-melatonin group. There is a highly significant difference presents between control and cisplatin group, there is also a significant difference between cisplatin-melatonin group and cisplatin group **(table 2, fig. 15).**

B) Surface area fraction of Mallory's trichrome positive stained liver tissues

There is a normal percentage of collagen fibers in the control and the melatonin groups but highly increase in the cisplatin group and markedly decreased in the cisplatin-melatonin group. There is a highly significant difference presents between the control and the cisplatin group, there is also a significant difference between the cisplatin group with the cisplatin-melatonin group **(table 3, fig.16).**

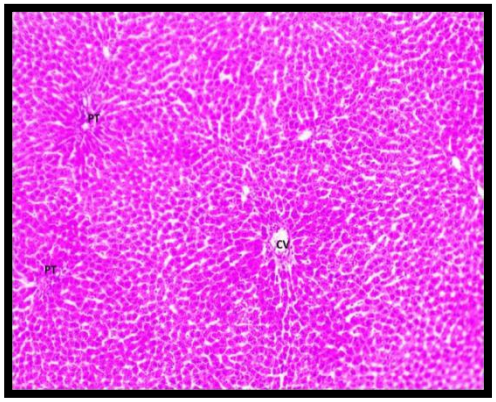

Fig. (1): A photomicrograph of rat liver tissue of group1 (control group),showing normal hepatic architecture. Notice the central vein (CV) in the center of the hepatic lobule with cords of hepato-cytes radiating from it and the portal tract (PT) at the periphery. (H&EX 100).

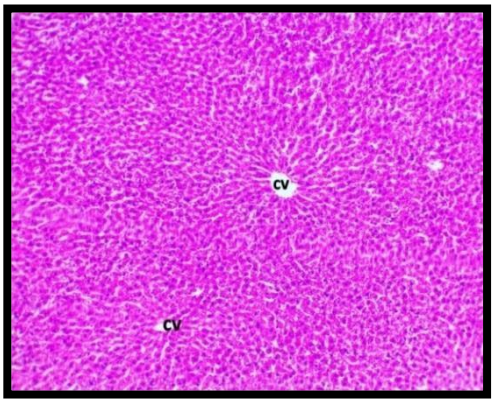

Fig. (2): A photomicrograph of rat liver tissue of group 2 (Melatonin) showing preserved hepatic architecture with central vein (CV) and portal tract (PT). (H&EX100).

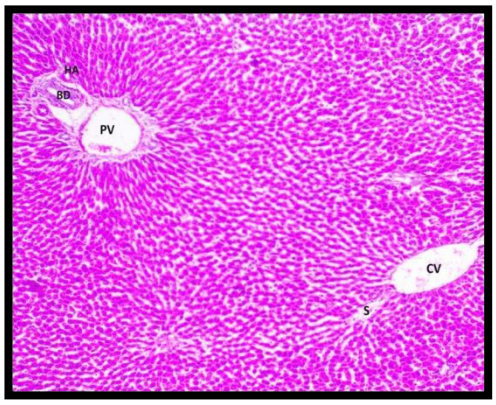

Fig. (3): A photomicrograph of rat liver tissue of group 3 (cisplatin) showing markedly dilated central vein (CV), portal tract (PT), disturbed hepatic architecture and dilated sinusoids (S) (H&EX100).

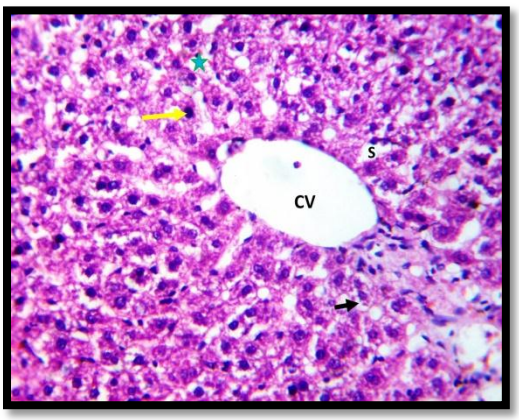

Fig. (4): A photomicrograph of rat liver tissue of group 3 (cisplatin) showing loss of normal lobular architecture with markedly dilated cental vein (CV). There is marked vaculation of hepatocytes (black arrow). Apoptotic cells appear with hypereosinophilic cytoplasm and darkly stained condensed nuclei (yellow arrow). There are dilated congested sinusoids (green star). (H&EX400).

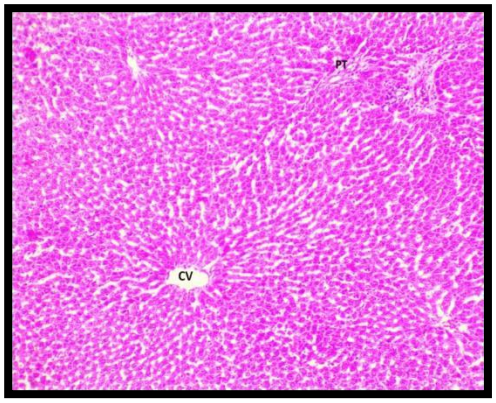

Fig. (5): A photomicrograph of rat liver tissue of group4 (Cisplatin+Melatonin) showing nearly restoration of hepatic lobular architecture, almost normal central vein (CV) and dimenshed inflammatory area around
it. (H&EX100).

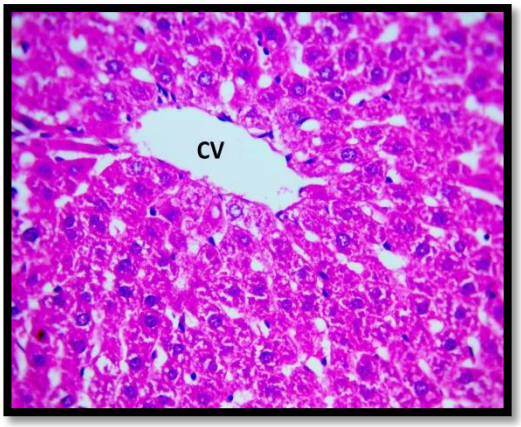

Fig. (6): A photomicrograph of rat liver tissue of group4 (Cisplatin+Melatonin) showing nearly restoration of hepatic lobular architecture, almost normal central vein(CV)and dimenshed inflammatory area around it with decreased vaculation of hepatocytes. (H&EX400).

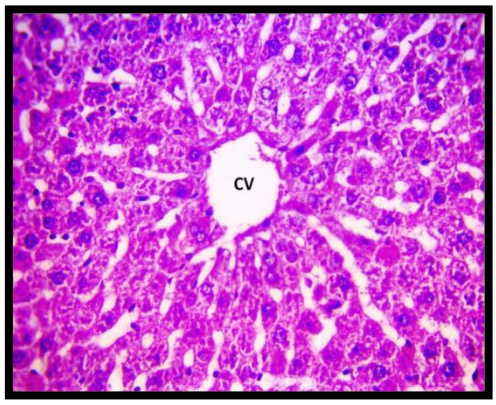

Fig. (7): A photomicrograph of rat liver tissue of group1(control group), showing positive deeply stained magenta color of glycogen granules in the cytoplasm of hepatocytes.(very strong PAS reaction) (PASX400).

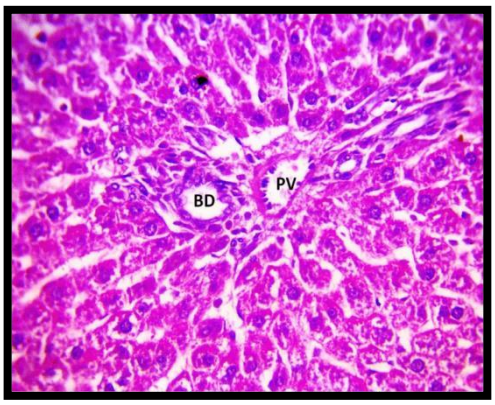

Fig. (8): A photomicrograph of rat liver tissue of group 2 (Melatonin) showing positive deeply stained magenta red glycogen granules in the cytoplasm of hepatocytes (very strong PAS reaction)(PASX400).

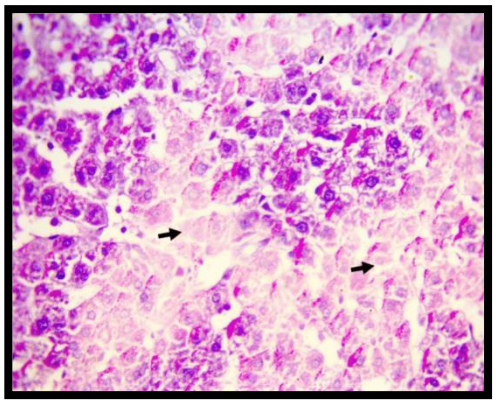

Fig. (9): A photomicrograph of rat liver tissue of group 3 (Cisplatin), showing a weak PAS reaction in the cytoplasm of hepatocytes. Other hepatocytes show no reaction (black arrows) (PASX400).

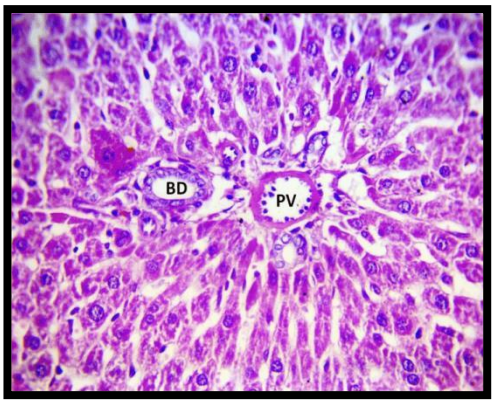

Fig. (10): A photomicrograph of rat liver tissue of group 4 (Cisplatin+Melatonin) showing relatively improvement of PAS reaction. (PASX400).

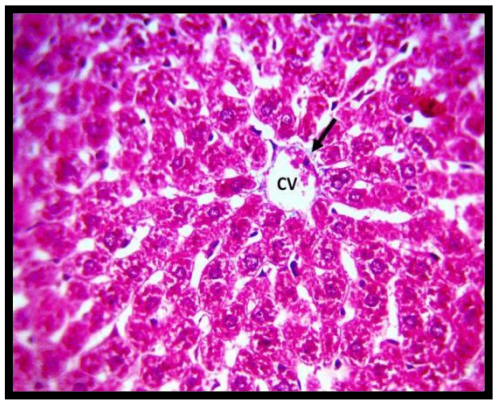

Fig. (11): A photomicrograph of rat liver tissue of group 1 (control group), showing minimal collagen fibers (arrow) around the central vein. (Mallory X400).

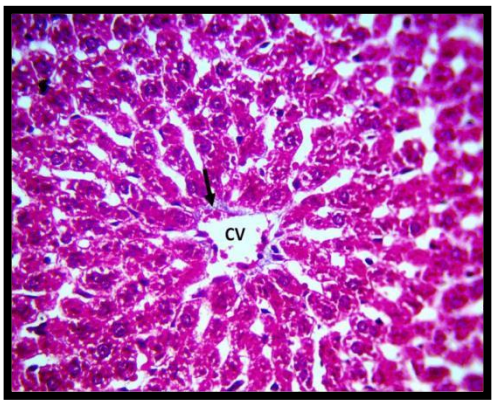

Fig. (12): A photomicrograph of rat liver tissue of group2(Melatonin) showing minimal collagen fibers (arrow)around the central vein. (Mallory X400).

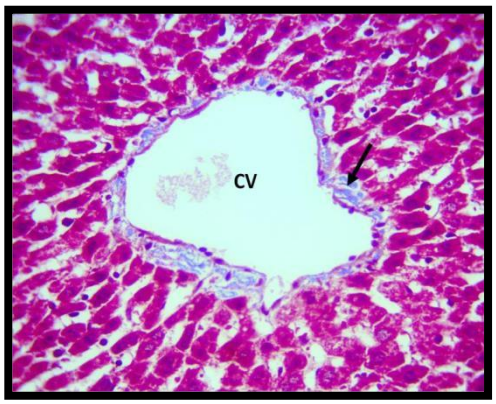

Fig. (13): A photomicrograph of rat liver tissue of group3 (Cisplatin), showing extensive collagen fibers accumulation around a dilated central vein (arrow) (MalloryX400).

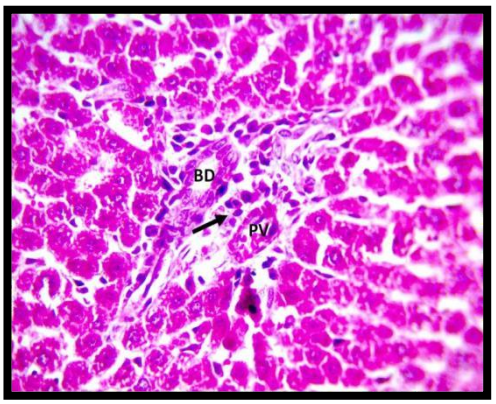

Fig. (14): A photomicrograph of rat liver tissue of group4 (Cisplatin + Melatonin) showing very minimal collagen fibers (arrow) around the portal tract.(MalloryX400).

Table (1): Serum AST& ALT levels in the different studied groups (IU/L).

	Group I (Control) (n=10)	Group II (Melatonin) (n=10)	Group III (Cisplatin) (n=10)	Group IV (Cisplatin+ (Melatonin) (n=10)	P. value					
AST Mean ± SD	115.3±3.1	120.7±2.7	185.9±3.4	140.4±3.2	<0.001*					
					P1	P2	P3	P4	P5	P6
					0.520	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
ALT Mean ± SD	35.9±5.1	38.4±3.6	84.1±2.9	63.7±2.6	<0.001*					
					P1	P2	P3	P4	P5	P6
					0.017*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

One Way ANOVA test for quantitative data between the four groups followed by Tukey post Hoc analysisbetween each two groups.*:Significant difference at P value <0.05.

P1 (g1-g2), P2 (g1-g3), P3 (g1-g4), P4 (g2-g3), P5 (g2-g4), P6 (g3-g4)

Table (2): showing the area fraction values of PAS stain (%)among the studied groups:

		Control	Melatonin	Cisplatin	Cisplatin + Melatonin	P value
		N=10	N=10	N=10	N=10	
Liver. PAS area fraction	Range Mean ± *SD*	(28.8-31.7) 33±1	(28.7-31.6) 32.1±1	(12-15) 14.8±0.9	(24.9-26.7) 26.6±0.6	***<0.001****
P. value between each two groups						
Control			**0.880**	**<0.001***	**<0.001***	
Melatonin				**<0.001***	**<0.001***	
Cisplatin					**<0.001***	

One Way ANOVA test for quantitative data between the four groups followed by post Hoc LSD analysisbetween each two groups
*: Significant level at P.value <0.05

Table (3): showing the area fraction values of Mallory's trichrome stain (%) Among the studiedgroups:

		Control	Melatonin	Cisplatin	Cisplatin + Melatonin	P. value
		N=10	N=10	N=10	N=10	
Liver-Mallory area fraction	Range Mean ± *SD*	(1.4-2.6) 2.5±0.4	(1.6-2.6) 2.5±0.3	(12.4-15) 13.9±0.9	(3.7-4.6) 4.3±0.3	***<0.001****
P. value between each two groups						
Control			*1*	***<0.001****	***<0.001****	
Melatonin				***<0.001****	***<0.001****	
Cisplatin					***<0.001****	

One Way ANOVA test for quantitative data between the four groups followed by post Hoc LSD analysisbetween each two groups
*: Significant level at P value<0.05

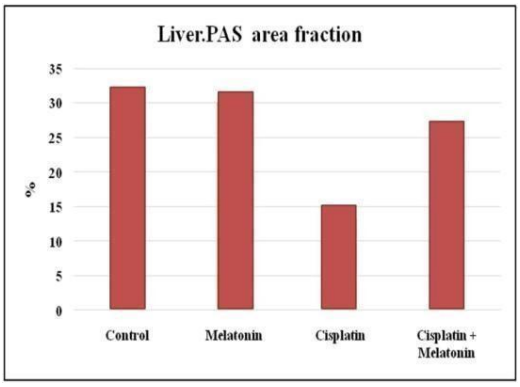

Figure15: Histogram showing the percentage ofPAS area fraction between different studied groups.

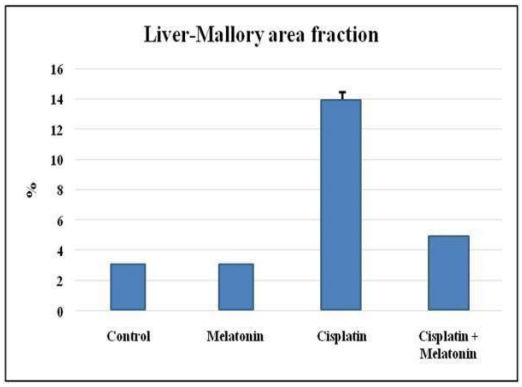

Figure16: Histogram showing the percentage of collagen fibers (fibrosis) between different studied groups

Discussion

Cisplatin is an anti-cancer drug used for solid organ tumors, it is a small molecule that can easily pass through cell membranes to enter the nucleus and change the DNA structure[22,23]. Melatonin is a hormone synthesized primarily by the pineal gland. Many studies had documented the efficacy of melatonin as a powerful antioxidant [10]. In order to reduce the liver damage caused by cisplatin, we used melatonin as a prophylactic agent in the present study.

Biochemical results of the current study showed that cisplatin produced hepatotoxicity after a single dose. The liver enzyme blood levels ALT and AST significantly increased, which was evidence of the hepatocellular injury in accordance to [24]. Co adminstration of melatonin significantly attenuates this elevation in hepatic enzymes. This finding was in agreement with other studies which reported that injection of melatonin for rats with liver diseases improved all liver enzymes which affected by hepatic damage[25].

By using of haematoxylin and eosin stained liver sections, the current study showed that cisplatin caused histopathological changes in liver architecture in the form of sinusoidal dilatation and haemorrhage, vacuolation of hepatocytes, dilatation and congestion of the central vein and the portal vein. Others studied the effect of single injection of cisplatin and found that CP treatment displayed noticeable liver impairments as revealed by histo-pathological and biochemical alterations with the escalation of liver function enzymes, reduction in antioxidant profile, hepatic oxidative damage and inflammatory reaction[26,27,28].

The main mechanism of cisplatin induced hepatotoxicity is induction of oxidative stress and apoptosis [29]. Oxidative stress results from overproduction of reactive oxygen species and the exhaustion of the natural antioxidant system. Oxidative stress caused damage to membrane lipids and other cellular component of the liver cells. Also the liver has the tendency to accumulate a significant amount of cisplatin, thus heptotoxicity and its histological alteration were associated with cisplatin administration [30].

Administration of melatonin with cisplatin showed the effectiveness of melatonin as the majority of the pathological alterations caused by cisplatin are improved. The liver cells appeared to be mostly normal, and the hepatic tissue has reestablished its normal structure, in parallel with [10] who reported that melatonin's capacity to scavenge free radicals decreased the oxidative stress condition that was common in rats given cisplatin.

Also melatonin caused down regulation of the expressions of pro-inflammatory markers as NF-κB and Cox-2 demonstrating the anti-inflammatory property of melatonin[31].

By using of Mallory's Trichrome stain, cisplatin increased amount of collagen fibers deposition. There was significant difference between cisplatin group and the control group. Others found that cisplatin significantly increased collagen fibers deposition by using Masson's trichrome stained sections[27].

In the present study, it was noticed that administration of melatonin in combination with cisplatin significantly decreased amount of collagen fibers deposition as compared to cisplatin group in agreement with other researches which stated that melatonin significantly reduced liver fibrosis induced by carbon tetrachloride through decreasing expressions of TGF-β1 and α-SMA expressions which are potent mediator cytokines for the synthesis of extracellular matrix and expansion of fibrosis[32].

According to PAS stain findings, cisplatin reduces the amount of glycogen present in hepatocytes as indicated by a weak PAS reaction and partial depletion in some regions. There was a significant difference between this group and the control group in agreement with other researchers who examined the rat liver after a single intraperitoneal injection of cisplatin and reported that the liver dysfunction was clarified by the morphometric analysis of PAS-stained liver sections to detect the significant decrease of PAS reaction in the liver of cisplatin group denoting minimal amount of stored glycogen as the synthesis and storage of glycogen granules are the functions of normal hepatocytes[26].

It was observed in the cisplatin- melatonin group that glycogen content started to be restored once again and improved PAS reaction with a significant difference between this group and the cisplatin group. Previous studies reported that melatonin treatment in rats conserved hepatic glycogen storage and also altered receptors for this polysaccharide through activation of protein kinase C and glycogen synthase kinase 3-dependent pathway by melatonin receptor[25]. Other studies found that melatonin improved PAS reaction in streptozotocin- induced diabetic rats[32].

Cisplatin had harmful effects on many organs including hepatotoxicity [33,34]. The present study showed that cisplatin can significantly induce hepatotoxicity in rats confirmed by biochemical and histopathological changes. Melatonin can attenuate the pathological effects of cisplatin.

Conclusion

Cisplatin caused liver injury mainly through oxidative stress (decreased natural antioxidants and increased reactive oxygen species, ROS), it caused destruction of tissue architecture and apoptosis. Melatonin therapy reduced oxidative stress and apoptotic activity, which improved liver function and pathological damage. Since melatonin is well tolerated by the body, it can be said that a pretreatment of it will undoubtedly be a promising support for chemotherapy as it scavenges the ROS and attenuates inflammation caused by cisplatin without interfering with its anticancer properties.

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