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Chemoprotective effect of melatonin against cisplatin-induced testicular toxicity in rats

Abstract: In this study, we investigated the effect of melatonin on cisplatin-induced spermiotoxicity using quantitative, biochemical and histopathological approaches. Cisplatin (CP, 7 mg/kg) and melatonin (10 mg/kg) were intraperitoneally injected. The rats were decapitated on 5th (short-term group) or 50th day (long-term group) after CP injection. Traits of reproductive organs, sperm characteristics, testicular histological findings, and the lipid peroxidation in the testicular tissue were determined. Melatonin mitigated CP-induced reductions in testes, epididymis and accessory gland weights in rats decapitated on day 5. Both short- and long-term CP treatment decreased sperm concentration, sperm motility and increased abnormal sperm rates compared with the control. But the reduction of sperm concentration in long-term CP treatment was insignificant. Although treatment with melatonin provided moderately normalization with respect to sperm concentration in short-term treatment group, melatonin caused a marked normalization of sperm motility in both CP + melatonin groups. Both groups treated with the melatonin showed decreases in abnormal sperm rates compared with alone CP. While testicular malondialdehyde levels were elevated after CP treatment, glutathione peroxidase activity decreased significantly in both groups. Glutathione levels reduced after long-term treatment, but not in short-term group by CP administration. Treatment with CP plus melatonin provided significant amelioration of oxidative stress parameters. Histopathological findings of testes in both short- and long-term treatment groups paralleled the biochemical and spermatogenic results. This study clearly indicates that CP-treatment impaired markedly testicular function and combined treatment with melatonin prevented much of the toxicity in rats.

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum-II, CP) is an effective antineoplastic agent in treatment of various solid tumours including cancers of the ovary, testis, bladder, head, neck, lung, cervix and endometrium [1]. Nevertheless, its full clinical utility is limited because of adverse effects on kidneys, peripheral nerves, the inner ear and testes, but the impairment of kidney function by CP is recognized as the main side effect and the most important dose limiting factor [2–5]. The biochemical basis of CP toxicity is believed to relate to free radicals generated in these tissues. The mechanism of testicular injury caused by CP is poorly understood; however, numerous studies have shown that CP exposure disrupts the redox balance of tissues suggesting that biochemical and physiological disturbances result from oxidative stress [6, 7]. Mammalian sperm cells present a highly specific lipid composition with high content of polyunsaturated fatty acids, plasmalogenes and sphingomyelins. This unusual structure of the sperm membrane is responsible for its flexibility and the functional ability. However, lipids in spermatozoa are the main substrates for peroxidation, which may provoke severe functional disorder of sperm [8, 9].

Biological compounds with antioxidant properties may contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species (ROS) and other free radicals induced by CP. Melatonin (*N*-acetyl-5-methoxytryptamine), originally discovered as a secretory product of the pineal gland, is a well-known antioxidant and free radical scavenger [10, 11]. The aims of this study were to investigate the effects of CP on sperm characteristics and biochemical, histopathological changes related with oxidative stress in testicular tissue and to highlight the possible protective effect of melatonin on these parameters.

Materials and methods

Chemicals

Cisplatin (10 mg/10 mL, Code 1876A) was purchased from Faulding Pharmaceuticals Plc (Warwickshire, UK); melatonin and the other chemicals were obtained from Sigma (St Louis, MO, USA).

Animals and treatments

In this investigation, 48 healthy adult male Sprague–Dawley rats (8 wk old, weighing 210–250 g) were used. The animals were obtained from the Frat University Medical School, Experimental Research Centre, Elazığ, Turkey. The animals were kept under standard laboratory conditions (12 hr light:12 hr dark and 24 ± 3°C). The rats were fed standard commercial laboratory chow (Elazığ Food Company, Elazığ, Turkey). Feed and water were provided ad lib. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health and were approved by the Local Committee on Animal Research.

The rats were randomly divided into two main groups for short- and long-term treatment; each group contained 24 rats. Then each main group was divided into four subgroups with each subgroup containing six rats. CP was injected intraperitoneally (i.p.) at the dose of 7 mg/kg, which is well documented to induce testicular toxicity in rats [12]. Melatonin was dissolved in ethanol and further diluted in saline to give a final concentration of 1% ethanol; this was administered i.p. at the dose of 10 mg/kg. Subgroup 1 (control) received a single dose i.p. injection of 1 mL isotonic saline. Subgroup 2 received melatonin for 5 days. Subgroup 3 received a single dose CP. Subgroup 4 received melatonin i.p. 24 hr prior to a single dose of CP and continued for 4 days after CP administration.

Sample collection

At the end of experimental periods, rats were decapitated on the 5th (short-term group) or 50th day (long-term group) after CP injection. Blood samples were collected into tubes containing sodium oxalate (2%) and centrifuged at 800 g for 10 min. Measurement of testes weight, length, thickness and epididymal, seminal vesicle and prostate weights were recorded along with epididymal sperm concentration, sperm motility and sperm morphology. One of the testes was fixed in 10% formalin for histopathological examination. Plasma and other testis were stored at −20°C until biochemical analyses.

Epididymal sperm concentration, motility and abnormal sperm rate

Spermatozoa in the right epididymis were counted by a modified method of Yokoi et al. [13]. Briefly, the epididymis was minced with anatomical scissors in 5 mL of physiological saline and was placed in a rocker for 10 min and then kept at room temperature for 2 min. After 2 min, the supernatant fluid was diluted 1:100 with a solution containing 5 g sodium bicarbonate, 1 mL formalin (35%) and 25 mg eosin per 100 mL of distilled water. Total sperm number was determined using a haemocytometer. Approximately 10 μL of the diluted sperm suspension was transferred to each counting chamber of the Improved Neubauer chamber and was allowed to stand for 5 min. The cells settled during this time were counted at 200× magnification in a light microscope.

The progressive motility was evaluated as described by Sönmez et al. [14]. For this purpose, a slide was placed on microscope stage and allowed to warm to a temperature of 37°C by means of a heater stage. Several droplets of Tris buffer solution [Tris (hydroxymetyl) aminomethane 3.63 g, glucose 0.50 g, citric acid 1.99 g and distilled water 100 mL] were then dropped onto the slide, and a very small droplet of fluid obtained from left cauda epididymis with a pipette was dropped on the Tris buffer solution and covered with a cover-slip. The percentage motility was evaluated visually at a magnification of 400×. Motility estimations were performed on three droplets and five different fields per drop in each sample. The mean value of five successive estimations was used as the final motility score.

The method by Ateşşahin et al. [12] was used for determination of the percentage of morphologically abnormal spermatozoa. Briefly, several droplets of Tris buffer solution was dropped on a clean, dry and prewarmed slide; then a small droplet of fluid obtained from left cauda epididymis with a pipette and covered two droplets of Indian ink were added on the Tris buffer + epididymal fluid solution and mixed by a cover-slip. A thin film of the stained sample was then drawn out onto another prewarmed slide. The slide was left to dry in a clean, dry and dust-free environment. After preparation, the slide was viewed under a light microscope at 400× magnification. A total of 400 sperm cells were examined on each slide, and the head, tail and total abnormalities of spermatozoa were expressed as percentage.

Biochemical assays

The testicular tissue was homogenized in Teflon-glass homogenizer with a buffer containing 1.5% potassium chloride to obtain 1:10 (w/v) whole homogenate. Concentrations of malondialdehyde (MDA), an index of lipid peroxidation (LPO), were measured in the homogenate. Then homogenates were centrifuged at 18,000 g (+4°C) for 30 min to determine reduced glutathione (GSH) levels. MDA concentrations were assayed according to a modified method of Ohkawa et al. [15] based on the reaction with thiobarbituric acid, and were expressed as nmol/g tissue. Glutathione peroxidase (GSH-Px) activity was determined as described by Lawrence and Burk [16]. The procedure of analysis was based on the oxidation of GSH by GSH-Px coupled to the disappearance of NADPH by glutathione reductase measured at 37°C and 340 nm and were expressed as IU/g protein. Tissue GSH concentrations were measured by a kinetic assay using a dithionitrobenzoic acid recycling method described by Ellman [17] and they were expressed as μmol/g tissue. Protein concentrations were measured according to Lowry et al. [18].

Histopathological examinations

Fixed testes in 10% formalin were embedded in paraffin, sectioned at 5 μm and were stained with haematoxylin and eosin. Light microscopy was used for the evaluations. The diameter and germinal cell layer thickness of the seminiferous tubule (ST) from five different areas of each testicle were measured using an ocular micrometer in a light

Table 1. Testes, epididymis, accessory gland weights (± S.E.M.) and testes dimensions

Organs	Length of treatment (days)	Control	Cisplatin	Melatonin	Cisplatin + melatonin
Testes weight (g)	5	1.29 ± 0.03[a]	1.16 ± 0.07[b]	1.30 ± 0.03[a]	1.25 ± 0.05[a]
	50	1.40 ± 0.03	1.26 ± 0.04	1.33 ± 0.03	1.29 ± 0.12
Testes length (cm)	5	1.84 ± 0.03	1.79 ± 0.15	1.91 ± 0.02	1.85 ± 0.02
	50	1.93 ± 0.02	1.87 ± 0.02	1.95 ± 0.02	1.94 ± 0.03
Testes thickness (cm)	5	0.99 ± 0.03	0.95 ± 0.02	1.02 ± 0.02	0.99 ± 0.01
	50	1.04 ± 0.01	1.00 ± 0.01	1.01 ± 0.03	1.00 ± 0.04
Epididymis weight (g)	5	0.40 ± 0.01[A]	0.26 ± 0.01[B]	0.40 ± 0.01[A]	0.34 ± 0.02[A]
	50	0.45 ± 0.01	0.38 ± 0.03	0.42 ± 0.03	0.38 ± 0.03
Seminal vesicle (g)	5	0.48 ± 0.08[A]	0.15 ± 0.01[B]	0.49 ± 0.09[A]	0.19 ± 0.02[B]
	50	0.60 ± 0.07	0.47 ± 0.08	0.56 ± 0.08	0.49 ± 0.04
Prostate (g)	5	0.25 ± 0.03[A]	0.11 ± 0.01[B]	0.24 ± 0.02[A]	0.21 ± 0.02[A]
	50	0.32 ± 0.04	0.20 ± 0.03	0.28 ± 0.09	0.23 ± 0.01

Different lower case ([a, b]$P < 0.05$) and upper case ([A, B, C]$P < 0.01$) letters within the same line are significantly different.

microscope, and the average size of ST and germinal cell layer thickness were calculated.

Statistical analyses

All values are presented as mean ± S.E.M. All groups were compared by one-way analyses of variance (ANOVA) and post hoc multiple comparisons were done with Duncan test in SPSS/PC software program (version 12.0; SPSS Inc., Chicago, IL, USA) to determine the differences in the all parameters.

Results

In the short-term treatment rats decapitated on day 5 after treatments had testes, epididymis, seminal vesicle and prostate weights that were significantly reduced in the CP group, with no changes in testes length and thickness when compared with the control group (Table 1). Although treatment with melatonin (CP + melatonin group) provided marked normalization in testes, epididymis and prostate weights when compared with CP alone, this treatment did not affect the seminal vesicle weights. However, in long-term treatment group rats after treatment, no statistical differences were observed in the reproductive organ weights or dimensions.

Cisplatin treatment for the short-term treatment caused a decrease in the epididymal sperm concentration, but reduction in the long-term group was not statistically significant (Fig. 1). Sperm motility reduced in both treatment group that received CP (Fig. 2), while increases in abnormal sperm rates also occurred in both groups (Table 2). Treatment with melatonin provided moderate normalization of sperm concentration in the short-term; the indole caused total recovery at both 5 and 50 days with respect to sperm motility. Both groups treated with melatonin also showed decreases in abnormal sperm rates compared with CP alone.

Cisplatin treatment caused significantly ($P < 0.01$) higher MDA levels (2.7-fold in short-term group, 1.6-fold in long-term group) in comparison with the controls (Table 3); these changes were practically reduced by melatonin (1.8-fold in short-term group, 1.5-fold in long-term group). CP caused significantly lower GSH-Px activity compared

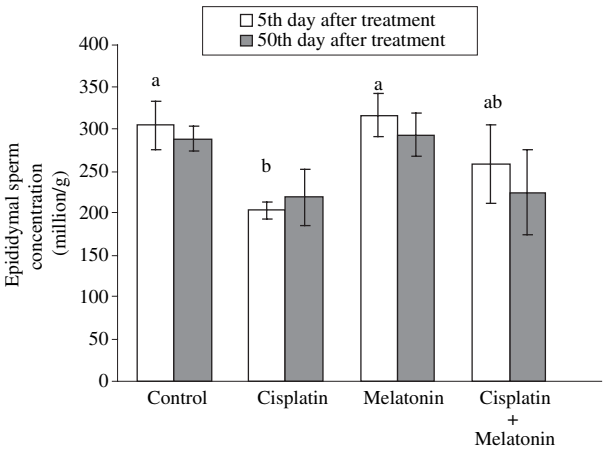

Fig. 1. Epididymal sperm concentrations (million/g).[a, b] Different lower case letters indicate significant differences ($P < 0.05$) among the groups.

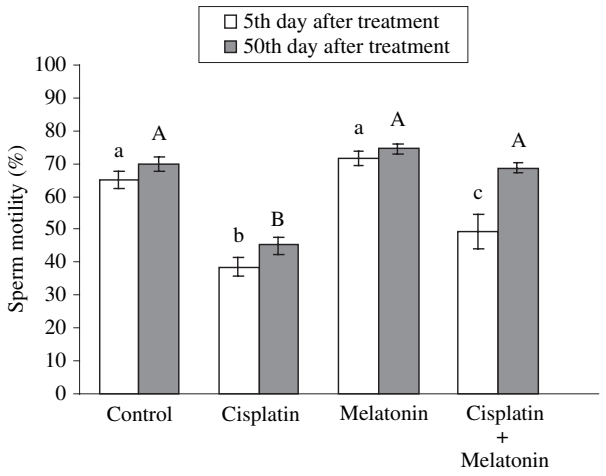

Fig. 2. Sperm motility rates (%).[a,b,c] Different lower case letters indicate significant differences ($P < 0.01$) among the short-term treatment groups.[A,B] Different upper case letters indicate significant differences ($P < 0.05$) among the long-term treatment groups.

Table 2. Abnormal sperm rates in rats were decapitated in after either 5 or 50 days of treatment

Portion of sperm	Length of treatment (days)	Control	Cisplatin	Melatonin	Cisplatin + melatonin
Head	5	5.45 ± 1.41	10.29 ± 0.75	5.05 ± 0.68	8.65 ± 2.87
	50	3.50 ± 0.42^{a}	10.95 ± 0.86^{b}	3.35 ± 0.35^{a}	7.00 ± 0.55^{ab}
Tail	5	2.12 ± 0.64^{a}	7.70 ± 0.44^{b}	1.98 ± 0.42^{a}	4.80 ± 0.90^{c}
	50	3.12 ± 0.21^{a}	9.00 ± 0.30^{b}	2.34 ± 0.26^{a}	4.60 ± 0.36^{ab}
Entire	5	7.57 ± 1.93^{A}	17.79 ± 0.71^{B}	7.03 ± 0.85^{A}	13.45 ± 3.69^{AB}
	50	6.62 ± 0.25^{A}	19.95 ± 0.46^{B}	5.69 ± 0.18^{A}	11.60 ± 1.52^{AB}

Different lower case ($^{a, b, c}P < 0.001$) and upper cases ($^{A, B}P < 0.05$) letter in the same line are significantly different.

Table 3. Testes tissue MDA, GSH and GSH-Px activities, and diameter size and germinal layer thickness

Parameter	Length of treatment (day)	Control	Cisplatin	Melatonin	Cisplatin + melatonin
MDA (nmol/mL)	5	0.65 ± 0.05^{a}	1.75 ± 0.22^{b}	0.68 ± 0.04^{a}	0.95 ± 0.09^{a}
	50	0.68 ± 0.06^{a}	1.12 ± 0.04^{b}	0.62 ± 0.03^{a}	0.72 ± 0.04^{a}
GSH (μmol/mL)	5	2.34 ± 0.16	2.81 ± 0.12	2.50 ± 0.11	2.45 ± 0.13
	50	3.28 ± 0.08^{a}	2.43 ± 0.12^{b}	3.20 ± 0.04^{a}	3.13 ± 0.16^{a}
GSH-Px (IU/g protein)	5	12.9 ± 1.06^{a}	6.7 ± 0.98^{b}	14.6 ± 1.45^{a}	13.3 ± 1.0^{a}
	50	20.0 ± 2.02^{a}	14.9 ± 2.10^{b}	23.3 ± 1.61^{a}	20.2 ± 1.70^{a}
Diameter of ST (μm)	5	239 ± 2.3^{a}	217 ± 2.1^{b}	240 ± 2.1^{a}	228 ± 2.1^{c}
	50	241 ± 2.0^{a}	185 ± 2.1^{b}	246 ± 2.0^{a}	187 ± 2.4^{b}
Germinal layer thickness (μm)	5	63.7 ± 0.69^{a}	54.4 ± 0.56^{c}	60.2 ± 0.66^{a}	58.7 ± 0.68^{b}
	50	68.7 ± 0.65^{a}	40.3 ± 1.02^{c}	71.4 ± 0.67^{a}	45.6 ± 0.96^{b}

MDA, malondialdehyde; GSH, reduce glutathione; GSH-Px, glutathione peroxidase; ST, seminiferous tubule.
$^{a, b, c}$Different letters within same line differ significantly ($P < 0.001$).

with the control group, only melatonin and CP + melatonin groups had higher activity of GSH-Px than CP alone at both treatment times. While there were no significant changes in the GSH levels among the subgroups after short-term treatment, there were significant reduction after long-term treatment with CP in comparison with the control; these reductions were normalized by melatonin (Table 3).

Upon microscopic examination, severe degeneration, necrosis and interstitial oedema were detected in short-term CP treatment group when compared with controls. The diameter of the STs and germinal cell thickness ($P < 0.001$) after CP were smaller than the controls (Table 2). Desquamative germinal cells were seen in ST lumen. The CP-induced changes in histopathological findings were partially reversed by treatment with melatonin (Fig. 3).

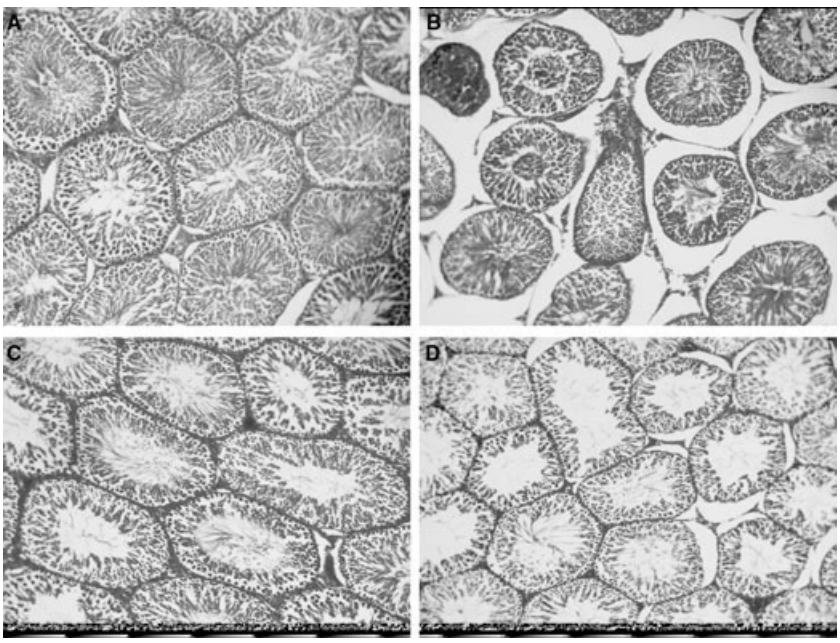

Fig. 3. Light microscopy of rat testes after short-term treatment with CP alone or with melatonin. Seminiferous tubules show severe degeneration, necrosis and interstitial edema in CP alone group. Seminiferous tubules also show degeneration and atrophy, but few necrotic changes and edema after CP plus melatonin treatment. A. Control, B. Cisplatin, C. Melatonin, D. Cisplatin + Melatonin. H & E, ×50.

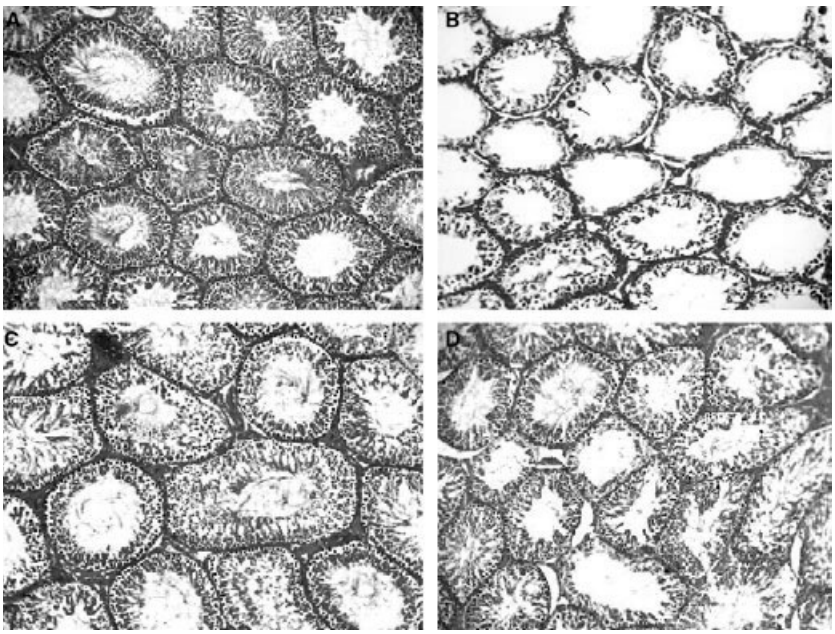

Fig. 4. Light microscopy of rat testes from long-term treament with CP alone or with melatonin. Syncytial cell formation was detected in ST, and these cells are shown with arrows. Treatment with melatonin ameliorated germinal cell thickness, but not ST diameter. In addition, administration of melatonin attenuated syncytial cell formation and atrophy seen in the CP treated rats, but same degenerative changes were maintained. A. Control, B. Cisplatin, C. Melatonin, D. Cisplatin + Melatonin. H & E, ×50.

In long-term treatment, CP caused degenerative changes and syncytial cell formation in the ST. Diameter and germinal cell thickness was also less in the control group. Treatment with melatonin ameliorated germinal cell thickness, but not diameter. In addition, administration of melatonin attenuated syncytial cell formation and severe atrophy seen in the CP group, but degenerative changes were maintained (Fig. 4).

Discussion

Effective anticancer therapy with cytotoxic drugs is limited by toxicity to kidneys, inner ear, peripheral nerves and testicular tissue [2, 10, 11]. Spermatogenic cells are targeted by cytotoxic agents because of their high mitotic activity. The chance of recovery of spermatogenesis following cytotoxic insult, and also the extent and speed of recovery, are related to the agent used and the dose received [19, 20].

It has been reported that administration of antitumor drugs to the male rats decreases reproductive organ weights. In the present study, administration of CP reduced testes, epididymis and accessory gland weights after short-term treatment when compared with control, confirming previous reports that CP decreases reproductive organs weights [21–23]. In addition, upon microscopic examination severe degeneration, necrosis and reductions in ST diameter and germinal cell thickness were observed after CP treatment. Reductions in testes weights are due to marked parenchymal atrophy in the ST of rats after CP administration. The accessory organs are dependent on testosterone produced by the interstitial cells. In this study, the reduction in accessory reproductive organ weights may be explained that accessory organs likely atrophied because these cells were damaged and testosterone levels were diminished. Melatonin partially attenuated the reduction in the weights at all organs measured after short-term treatment. In long-term treatment animals, CP did not significantly influence organ weights or dimensions.

Gwayi and Bernard [24] reported that melatonin receptors have been identified in epididymis and low-affinity melatonin-binding sites have been described on spermatozoa; hence, it is possible that melatonin influences sperm motility as they transit through the epididymis. Furthermore, abnormalities in sperm motility seem to be overcome by melatonin.

In the present study, administration of CP decreased significantly epididymal sperm concentration (short-term treatment) and motility, confirming several reports [12, 21]. The biochemical basis of CP toxicity is believed to relate to free radicals generated in testicular tissue. Mammalian spermatozoa are rich in polyunsaturated fatty acids and, thus, are susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased midpiece morphological defects. The recently described beneficial effect of melatonin at the mitochondrial level, which includes stimulatory effects of the indole on ATP production, may account for the preservation of sperm motility by melatonin [25]. LPO of the sperm membrane is considered to be the key mechanism of ROS-induced sperm damage leading to infertility [8, 9]. In this study, CP increased the testicular MDA levels, compared with the control values. CP-induced reduction in the number of sperm in the short period may be explained by the fact CP increased LPO levels or the drug damaged directly the mechanism of the spermiation. The significant reduction in sperm motility may be due to the toxic effect of CP on flagellum, important machinery for motility or sperm cells. Some investigators [25, 26] reported that ATP is an energy source for sperm motility and its availability may be limiting factor responsible for loss of sperm motility in CP-treated rats. As note above melatonin promotes ATP production [25]. Significant increases were observed in the CP + melatonin group rats with respect to sperm concentration (only short-term group) when compared with CP alone. Similarly, melatonin prevented the

CP-induced reductions in sperm motility in both short- and long-term treatment groups. Increases in sperm concentration, sperm motility and relative amelioration in histological structure of testes in the CP + melatonin group compared with the CP-alone group probably relates to the free radical scavenging ability of melatonin.

The spermatogenic cycle includes spermatogenesis, meiosis and spermiogenesis which require 48–52 days in rats [27]. In this study, we selected the 50-day period for evaluation of side effects of CP on the spermatogenic cycle. After 50 days, sperm concentration was insignificantly decreased by CP. The only slightly negative effect of CP on sperm concentration after long-term treatment may be that CP affected only moderately mitotic and meiotic divisions in the spermatogenic cycle during this period.

The results of the present study indicated that CP administration caused increases in abnormal sperm. Cellular DNA is a primary target of CP in its anticancer and toxic activity [28]. Also, its mutagenic potential has been shown in bacteria and mammalian cells. Increases in oxidative stress probably play a critical role in the induction of sperm abnormalities, namely a higher susceptibility of sperm DNA to denaturation and fragmentation [8, 29]. CP has physiological side effects leading to mutations and other genotoxic changes in nontumour cells. The causes of increases in abnormal sperm may be because of its genotoxic and mutagenic effect on germ cells. Melatonin prevented the CP-induced increases in the abnormal sperm. Again, rationale mechanism for the antimutagenic effects of melatonin is its ability to scavenge free radicals that cause oxidative DNA damage.

Reduced glutathione is the major cellular sulphydryl which serves as both nucleophile and an effective reductant by interacting with numerous electrophilic and oxidizing compounds. It is important in the regulation of the cellular redox state and a decline in its cellular level has been considered indicative of oxidative stress [30, 31]. Increases and/or decreases in the GSH levels are apparent after several chemotherapeutic agent exposures. The increased concentration of GSH in the testicular tissue supports the idea of ROS involvement in our experimental conditions. GSH synthesis is induced in cells exposed to oxidative stress as an adaptive process. In the present study, treatment with melatonin in part reversed the increase in the GSH levels.

In an attempt to protect sperm function from CP several antioxidants have been tested [10, 12, 26, 28]. Melatonin is certainly a potential ameliorative agent against the damage caused by CP. This antitumor drug generates active oxygen species, such as superoxide anions and hydroxyl radicals in testicular tissue. It has been reported that melatonin can directly neutralize these free radicals and related toxicants [10, 32, 33]. Tan et al. [34] showed that each melatonin molecule actually scavenges two $^{\bullet}OH$. Unlike some other well-known antioxidants which are exclusively lipid (e.g. vitamin E) or water-soluble (e.g. vitamin C) and therefore exhibit a limited intracellular distribution, melatonin is amphilic allowing it to reduce free radical-mediated damage in both the lipid and aqueous subcellular compartments. Hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{-\bullet}$) and peroxyl radical ($LOO^{\bullet}$) are scavenged by melatonin [10, 33, 34]. Melatonin has been shown to decrease MDA and increase GSH levels in several pathological conditions such as doxorubicin or CP toxicity [11, 35]. Besides melatonin, metabolites (N^1-acetyl-5-methoxynuramine) are formed when melatonin detoxifies radicals and radical products are also highly effective free radical scavengers [36–38]; this greatly increases the antioxidant properties of melatonin.

In conclusion, this study clearly indicates that CP treatment markedly impairs testicular function and treatment with melatonin in part prevents this toxicity in rats. Our results also suggest that melatonin may have a significant effect for clinical applications such as impairment of testicular function that is induced by cytotoxic chemotherapeutics.

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