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Effect of Melatonin on Doxorubicin Induced Testicular Damage in Rats

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Abstract: The protective effect of melatonin against doxorubicin induced testicular toxicity was investigated in male rats. Melatonin was administered orally to rats at a dose of 6 mg/kg daily for 28 days along with doxorubicin. Doxorubicin was administered intraperitoneally to the animals at a dose of 3mg/kg once a week for 5 weeks (a total of 15mg/kg) on day 1, 7,14,21,28. Treatment with doxorubicin alone caused decrease in body weight, sperm count and serum testosterone and increase in serum levels of lactate dehydrogenase (LDH), creatine phosphokinase (CK), and glutamic oxaloacetate transaminase (GOT). However, the combined treatment of melatonin with doxorubicin restored the body weight, sperm count and serum markers to normal. It was observed that administration of melatonin along with doxorubicin caused significant increase in superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH), membrane bound enzymes like Na^+K^+ATPase, Ca^{2+}ATPase, Mg^{2+}ATPase and decrease in lipid peroxidation (LP) in testes, indicating protection afforded by melatonin administration. These findings indicate that melatonin might be having protective effect against doxorubicin induced testicular toxicity.

Keywords: Melatonin, Doxorubicin, Antioxidant, Testicular toxicity

Introduction

Doxorubicin, an anthracycline antibiotic, is a widely used anticancer agent. Inspite of its high antitumor efficacy, the use of doxorubicin in clinical chemotherapy is limited due to diverse toxicities, including testicular toxicity [1, 2, 3]. Although a number of potential toxic mechanisms have been identified following exposure to doxorubicin, the major pathogenic mechanism appears to involve the generation of toxic reactive oxygen species [4]. Doxorubicin induced toxicity has been alleviated by administering various natural and artificial compounds [5, 6, 7]. Melatonin (*N*-acetyl-5-methoxytryptamine), the chief hormone of the pineal gland, attracted much interest after its antioxidant ability was proven by both *in vivo* and *in vitro* studies [9, 10]. Melatonin acts as a powerful antioxidant and as a free radical scavenger of hydroxyl, peroxyl radicals, and superoxide anions [11]. Indeed, Melatonin was shown to be twice as potent as vitamin E in removing peroxyl radicals [12] and it is more effective in scavenging hydroxyl radicals than glutathione and mannitol [13]. The direct effects of this hormone on the male reproductive system have also been examined in several animal studies. Since melatonin binding sites were detected in the reproductive system of different species [14, 15], it seems reasonable to assume that melatonin exerts its actions via direct interaction with the steroidogenic cells of the reproductive organs.

Damage to the testicular germinal epithelium is a potential side effect of cancer therapy, and is of particular concern in case of men of reproductive age having tumors with high cure rates [16]. Therefore, the aim of present study was to evaluate the protective effect of melatonin on doxorubicin induced testicular damage by using serologic, histopathologic, and biochemical analyses.

2. Material and methods

2.1. Chemicals

Doxorubicin injection was obtained as a gift sample from Serum Institute of India Ltd., Pune. Melatonin, Super

oxide dismutase (SOD), Malondialdehyde, Catalase, were purchased from Sigma Aldrich; USA. Reduced glutathione, 5, 5'Dithiobis (-2 nitrobenzoic acid) (DTNB), Thiobarbituric acid (TBA) from Hi Media; India. All other chemicals were of analytical grade.

2.2. Animals

Male albino rats (Wistar strain) weighing between 200 and 250 g were used for the study. The animals were fed *ad libitum* with standard pellet diet and had free access to water. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda and are in accordance with guidelines as per "Guide for the care and use of laboratory animals" published by NIH publication (NO 85-23 revised 1996) and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.3. Experimental Protocol

The animals were divided into four groups each consisting of six rats and received the following treatment.

Group I (Control): received vehicle (0.5%CMC, 2 ml/kg /day p.o. for 28 days) and sterile water for injection (1ml/kg, i.p.) on day 1, 7, 14, 21, 28.

Group II (Doxo): Doxorubicin injection (3 mg/kg i.p.) on day 1, 7, 14, 21, 28.

Group III (Doxo+Mel): Melatonin (6mg/kg /day p.o. for 28 days) and doxorubicin injection (3 mg/kg i.p.) on day 1, 7,14,21,28.

Group IV (Melatonin): Melatonin (6mg/kg /day p.o.) for 28 days.

After 48 hours of the last injection of either doxorubicin or vehicle, blood was collected for serological analyses. Epididymis was removed, cleared off the adhering tissues and weighed. The epididymal sperm count was done immediately. The testes was excised under euthanasia in chilled Tris buffer (10 mM pH 7.4) for measurement of tissue markers of oxidative stress the other one was collected for histopathology.

Epididymal sperm count: Epididymal sperm was collected by slicing the epididymis in 5 mL phosphate buffered saline (pH 7.2) [10]. An aliquot of the epididymal sperm suspension was used for spermatozoa count using Neubauer hemocytometer [17].

Serological analyses: Serum levels of lactate dehydrogenase (LDH) and serum creatine kinase (CK) were determined by using standard kits of Reckon Diagnostic Ltd, India while glutamic oxaloacetate transaminase (SGOT) was estimated by using standard kit of Span Diagnostic Pvt Ltd, India. Testosterone level was estimated by direct chemiluminescent assay (ADVIA CENTAUR).

Biochemical analyses: The excised testes was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000×*g* at 0° C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assays of malondialdehyde content as indicator of lipid peroxidation (LP) [18] endogenous antioxidant enzymes, superoxide dismutase (SOD) [19], catalase (CAT) [20] and reduced glutathione (GSH) [21]. The sediment after centrifugation of tissue homogenate was resuspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes such as Na^+K^+ATPase [22], Ca^{2+}ATPase[23]and Mg^{2+}ATPase [24] and Total proteins [25].

Histopathologic examination: For histotological evaluation, the testes were fixed in 10% formalin, dehydrated and embedded in paraffin. Tissues were then sectioned at 4 μm, stained with haematoxylin and eosin (H&E) and examined for histopathological evidence under Olympus BX40 Photomicroscope.

2.4. Statistical analysis

Results of all the above estimations have been indicated in terms of mean ± S.E.M. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey –Kramer multiple Comparisons test with the level of significance set at $P \leq 0.05$.

3. Results

3.1. Body weight, testes weight and sperm count: All animals survived the experimental period. Administration of doxorubicin alone significantly reduced body and testes weight as compared to control animals. Administration of melatonin along with doxorubicin restored body and testes weight to normal. Administration of doxorubicin alone significantly decreased sperm count while it was significantly increased with melatonin coadministration (Table 1).

3.2 Serological analyses: Administration of doxorubicin alone significantly increased serum level of CK, LDH and GOT and decreased the level of testosterone as compared to control rats. The administration of melatonin along with doxorubicin significantly restored serum marker levels towards the control value (Table 2).

3.3 Biochemical analyses: Administration of doxorubicin alone significantly increases LP while there was a significant decrease in GSH, SOD and CAT levels as compared to control rats. Administration of melatonin along with doxorubicin significantly restored GSH, SOD, CAT and LP levels towards control value. Administration of doxorubicin alone significantly decreased the levels of membrane bound enzymes like Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase as compared to control. Administration of melatonin along with doxorubicin significantly restored membrane bound enzyme levels towards control value (Table 3 and 4).

3.4. Histopathologic findings: Doxorubicin causes vacuolization and fibrinoid debris in the seminiferous tubules. Shrunken seminiferous tubules showed loss of germ cell. Widening of the interstitial space and severe

vacuolization were also observed in interstitial tissues. Administration of melatonin along with doxorubicin restored these changes towards normalcy (Fig.1).

4. Discussion

Many drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organ systems including the testes. A strategy to diminish the side effects of anticancer drugs with preservation of their chemotherapeutic efficacy is necessary. Doxorubicin is known to disturb spermatogenesis in a dose-dependent manner in animal studies [3]. With a low dose of doxorubicin, a significant but temporary reduction in spermatogenesis occurred [26]. Ward et al. [27] also reported that doxorubicin induced reductions in testicular sperm count. In the present study, male rats were treated with a total of 15 mg/kg of doxorubicin for 5 weeks. The results indicated above that doxorubicin induce pathological changes in serum and biochemical markers indicative of toxicity and increases the free radical production. These results were consistent with earlier studies [3, 27, 28]. Elevated serum levels of LDH, CK and GOT suggest that doxorubicin may induce generalized toxicity in rats.

Further results also led to belief that administration of melatonin improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of LP. The restoration of membrane bound enzymes like Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase in melatonin treated rats was due to the membrane stabilizing protective effect of melatonin [29]. These protective effects were also supported by the restoration of sperm count, histopathologic study and serum marker of toxicity.

Experimental studies have shown that melatonin directly scavenges the hydroxyl radical, peroxyl radical, peroxynitrite anion, and singlet oxygen. Furthermore, this tryptophan derivative stimulates a number of antioxidative enzymes and stabilizes cell membranes; this action helps membranes to resist free radical damage. It has been reported that melatonin protects doxorubicin induced cardiac injury by inhibiting necrosis and apoptosis, thereby improving doxorubicin damaged cardiac function. The exact mechanism of cardioprotective effect needs to be explored further; however, because melatonin do not compromise the antitumor effect of doxorubicin, the combined treatment of doxorubicin and melatonin holds promise as a safe and effective chemotherapeutic strategy [30].

In conclusion, melatonin was found to be a potential candidate as an additive to chemotherapeutic agents that are toxic to testes. Melatonin could be an effective regimen to enhance therapeutic efficacy and to reduce toxic side effects of doxorubicin in clinical chemotherapy.

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Table 1: Effect of doxorubicin alone and along with melatonin on body weight, testes weight and sperm count.

Groups	Final body weight (BW, g)	Absolute testes weight (g)	Relative testes weight (per BW, %)	Sperm Count ($x10^6$/mg epididymis)
Control (Group I)	227.5± 4.23	2 ± 0.085	0.87±0.041	15.7±0.47
Doxo (Group II)	204.2±4.16*	1.5±0.081**	0.73±0.024*	11.38±0.91**
Doxo+Mel (Group III)	220±4.28^{NS}	1.66±0.084^{NS}	0.75±0.038^{NS}	13.85±0.36*
Melatonin (Group IV)	228.3±6.66	1.95±0.076	0.85± 0.022	14.55±0.38
P value	0.0086	0.00085	0.011	0.00031
F value	5.11	8.34	4.73	9.99

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, **NS** = Non significant

Table 2: Effect of doxorubicin alone and along with melatonin on serum levels of LDH, CK-NAC, SGOT and Testosterone.

Groups	LDH (U/L)	CK-NAC (U/L)	SGOT (U/ml)	Testosterone (ng/ml)
Control (Group I)	169.83 ± 4.62	231.16±12.68	32.33 ± 2.0	0.80±0.045
DOX (Group II)	610.33±77.66***	511.5±17.69***	102.05±5.86***	0.63±0.061NS
DOX+MEL (Group III)	334 ±18.42***	328.3 ± 4.96***	62.08 ± 7.98***	0.69± 0.038NS
Melatonin (Group IV)	185 ± 7.638	241.7 ± 14.93	36.67 ±3.33	0.78± 0.049
P Value	<0.0001	<0.0001	<0.0001	0.064
F Value	25.87	51.03	36.14	2.83

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant.

Table 3: Effect of doxorubicin alone and along with green tea extract on biomarkers of the oxidative stress.

Groups	LP (nmolesof MDA/mg protein)	GSH (µg of GSH/ mg protein)	SOD (Units/mg protein)	CAT (µmoles of H_2O_2 consumed/min/ mg protein)
Control (Group I)	1.22 ± 0.043	4.15±0.32	4.92 ± 0.97	7.29±0.77
DOX (Group II)	1.82 ± 0.10***	2.63±0.15***	2.55 ± 0.15*	3.90±0.27***
DOX+MEL(Group III)	1.44±0.1*	3.68±0.069*	3.32±0.16NS	6.38±0.36**
Melatonin (Group IV)	1.13 ±0.05	4.22±0.37	4.71±0.19	7.27±0.34
P Value	<0.0001	0.001164	0.00994	0.00018
F Value	13.8	7.86	4.94	10.95

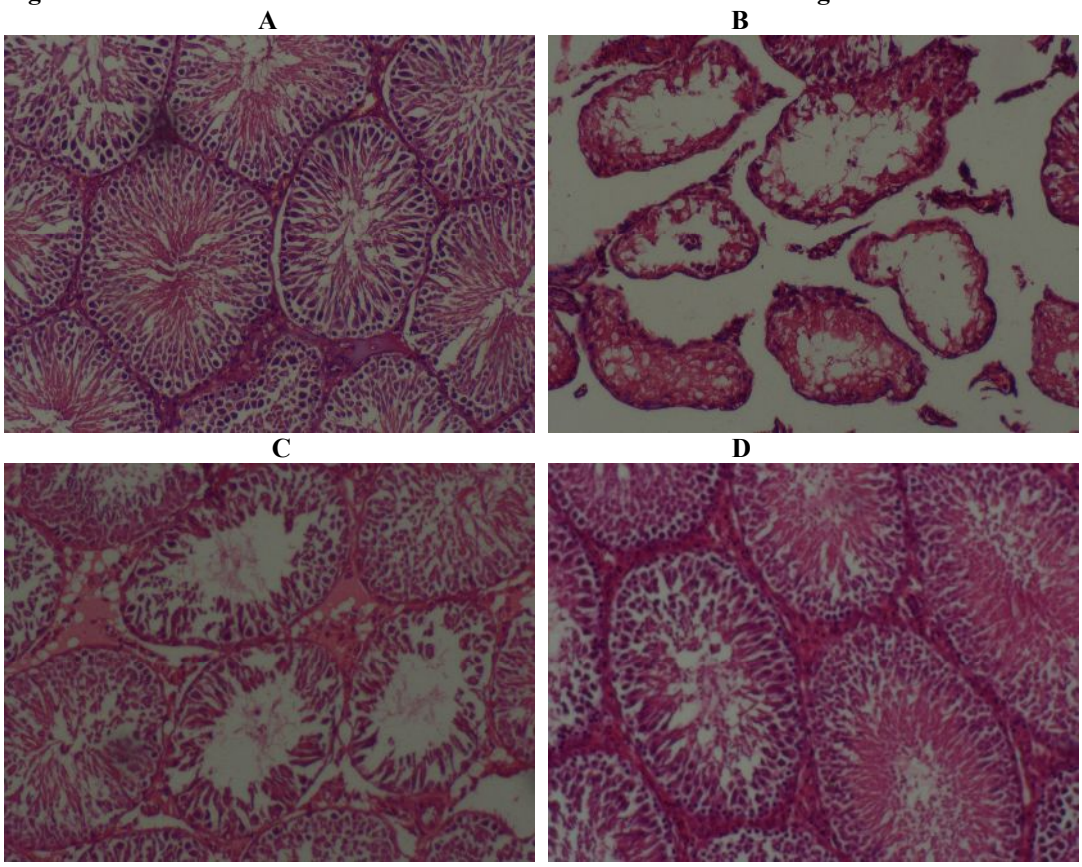
Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant

Table 4: Effect of doxorubicin alone and along with green tea extract on membrane bound enzymes.

Groups	Na^+K^+ATPase (µmoles of inorganic phosphrous liberated /min /mg protein)	Ca^{2+}ATPase (µmoles of inorganic phosphrous liberated /min/ mg protein)	Mg^{2+}ATPase (µmoles ofinorganic phosphrous liberated / min/ mg protein)
Control(Group I)	8.50±0.34	4.24±0.57	6.56±0.40
DOX(Group II)	5.01±0.30***	3.08±0.22NS	4.01±0.26***
DOX+Mel (Group III)	6.82±0.42*	3.98±0.31NS	5.49±0.23**
Melatonin (Group IV)	7.85±0.48	4.16±0.55	6.33±0.21
P Value	<0.0001	0.26	<0.0001
F Value	14.79	1.43	15.99

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Not significant

Fig. 1: Cross sections of testes in rats treated with doxorubicin and along with melatonin.



A: Control, **B**: Doxorubicin alone, **C**: Doxorubicin + Melatonin, **D**: Melatonin alone
Testes from control (A) and melatonin treated rats (D) shows normal feature of seminiferous epithelium and interstitial tissue. However, testes from a doxorubicin treated rats (B) reveals markedly shrunken and empty seminiferous tubules. Administration of melatonin along with doxorubicin (C) restored these changes towards normalcy.

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