ATTENUATION OF CISPLATIN ASSOCIATED TESTICULAR TOXICITY BY 6-HYDROXYFLAVONE

Zia Ud Din[1], Shabnum Amir[1], Muhammad Haris[1], Naila Ismail[2], Noman Ullah Wazir[3] Muhammad Shahid[4,5], Rahim Ullah[5], Nisar Ahmad[5,6], Umar Farooq[4], Ihsan Ullah[7]

[1]Department of Anatomy, Khyber Medical College, Peshawar, Khyber Pakhtunkhwa, Pakistan

[2]Department of Pathology, Khyber Medical College, Peshawar, Khyber Pakhtunkhwa, Pakistan

[3]Department of Anatomy, Peshawar Medical College, Peshawar, Khyber Pakhtunkhwa, Pakistan

[4]Department of Pharmacy, Sarhad University of Science and Information Technology, Peshawar, Khyber Pakhtunkhwa, Pakistan

[5]Department of Pharmacy,University of Peshawar, Khyber Pakhtunkhwa, Pakistan

[6]Department of Pharmacy, Abasyn University, Peshawar, Khyber Pakhtunkhwa, Pakistan

[7]Department of Pharmacy, University of Swabi, Khyber Pakhtunkhwa, Pakistan

ABSTRACT

Objectives: *Cisplatin is an effective anticancer drug for the treatment of various malignancies. However, cisplatin use is associated with different adverse effects including its toxicological propensity for testicular damage. Flavonoids have shown to possess strong antioxidant property and have a protective influence on human body. In the present study, the flavonoid, 6-hydroxyflavone was investigated against cisplatin associated testicular toxicity in rats.*

Materials & Methods: *The animals were daily treated with 6-hydroxyflavone at doses of 25 and 50 mg/kg for consecutive 15 days, while cisplatin was injected as a single dose (7.5 mg/kg, i.p.) on day 10 of the experiment. The testicular tissue was collected and malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) were measured. The tissues were also processed for histopathological evaluation.*

Results: *Cisplatin produced marked oxidative stress (increased MDA and decreased GSH and SOD) and caused degenerative changes in the germinal epithelium of the seminiferous tubules. Daily treatment with the tested doses of 6-hydroxyflavone attenuated the cisplatin-induced oxidative stress in the testes by significantly decreasing the levels of MDA, and increasing the contents of GSH and activity of SOD. Moreover, 6-hydroxyflavone also protected the germinal epithelium from the toxicological assertion of cisplatin as no significant histopathological aberrations were observed.*

Conclusion: *These finding suggest that flavonoids in general and 6-hydroxyflavone in particular may be used for the management of anti-cancer drugs associated adverse-effects particularly for their propensity towards reproductive system.*

Key Words: *6-Hydroxyflavone, Chemotherapy induced testicular damage, Flavonoids and testes, Anti-fertility, Natural products.*

Correspondence:
Dr. Zia Ud Din
Department of Anatomy, Khyber Medical College, Peshawar 25000, Khyber Pakhtunkhwa, Pakistan
Email: ziauddin003@gmail.com
Contact: +923329146044

INTRODUCTION

Cisplatinis a platinum compound. It is the main drug for the treatment of malignancies including testicular carcinoma, medulloblastoma, osteogenic sarcoma, and ovarian cancer.[1] Cisplatin exerts its

anticancer activity by introducing toxic DNA interstrand crosslinks into proliferating cells and inducing apoptosis (programed cell death) with different binding sites like guanine-N7 by causing both cytostatic and cytotoxic effects.[2] The chemotherapeuticcombination of cisplatin and 5-flourouracil has shownefectiveness in patients diagnosed with progressive squamous cell oral carcinoma and finallyadvances the overall survivability of patients.[3] Although cisplatinpossesses a beneficial chemotherapeutic effect; however, it has been associated with serious adverse effects on the vital organs. Cisplatin causes a gradual decrease in renal function and increases hemoglobin level,red blood cell count and eventually hematocrit.[1] Animals administered with cisplatin develop severe damage to the testicular tissue and is characterized by apoptosis of germ cells, dysfunction of Leydig cell, and testicular steroidogenic disorder.[4] Cisplatinalso affects the formation of sperms by inhibiting the synthesis of nucleic acid in the germ cells and also stop the production of testosterone by damaging the Leydig cells, and thus leading to the development of infertility.[5]

Flavonoids are an important group of natural compounds widely present throughout the plant kingdom.[6] Flavonoids have potent beneficial effects on the human health and present an indispensable constituent in a variety of daily use products including nutraceuticals, pharmaceuticals, and cosmetics.[7] The effectiveness of flavonoids has been attributed to their potent biological properties including strong free radical scavenging, ability to attenuate inflammation, inhibition of mutagenesis and carcinogenic growth along with their ability to produce modulation in key cellular enzyme function.[8] In view of the promising properties offered by flavonoids, in this study, the flavonoid, 6-hydroxyflavone was investigated in an animal model for its therapeutic potential against cisplatin associated testicular toxicity.

MATERIALS AND METHODS

Chemicals

6-Hydroxyflavone and ascorbic acid were procuredfromSigma-Aldrich, St. Louis, MO, USA. The 6-hydroxyflavone was dissolved in a vehicle consisting of 5% DMSO and 2% Tween80.[9,10] Ascorbic acid was dissolved in normal saline.[11] The drugs solutions were freshly prepared daily before intraperitoneal injection to the animals.

Animals

Male Sprague-Dawely rats weigh up 200-250 g were used in the current study. The animals were accustomed at a light dark cycle of 12/12 h at 20-24°C. All the experimental protocols were approved by the Ethical Board of Khyber Medical College, Peshawar, Pakistan and a registration number of 42/PG/KMC was obtained.

Grouping and dosing schedule

6-Hydroxyflavone (25 and 50 mg/kg) and ascorbic acid (50 mg/kg)[12] were administered through anintraperitoneal route (i.p.). The testicular toxicity was induced by a single injection of cisplatinat a dose of 7.5 mg/kg (i.p.).[13] The animals were randomly divided into following setsand each set consisted of six animals:

Group A attended as negative control and was injected with the vehicle (1 mL/kg, i.p.).

Group B received cisplatin as a single injection (7.5 mg/kg, i.p.) on day 10 of the experiment.Group C received daily 6-hydroxyflavone injection at 25 mg/kg for consecutive 15 days along with cisplatin (7.5 mg/kg, i.p.), which was administered two hours after 6-hydroxyflavone administration on day 10 of the experiment.

Group D received daily 6-hydroxyflavone injection at 50 mg/kg for consecutive 15 days along with cisplatin (7.5 mg/kg,i.p.), which was administered two hours after 6-hydroxyflavone administration on day 10 of the experiment.

Group E received daily ascorbic acid injection at 50 mg/kg for consecutive 15 days along with cisplatin (7.5 mg/kg, i.p.), which was administered two hours after 6-hydroxyflavone administration on day 10 of the experiment.

Group F received daily 6-hydroxyflavone injection at 50 mg/kg for successive 15 days.

Histopathological and biochemical analysis

At the end of dosing schedule, each animal was givenxylazine plus ketamine anesthesia. The testes were dissected out and were instantaneously transferred to a jar containing 10% neutrally buffered formalin.After testes collection, the animals were euthanized by cervical dislocation. A small portion of the testes were used for measuring the tissue

antioxidant status, while the remaining portion of testes were kept in buffered formalin for 48 hours.

For the assessment of malondialdehyde (MDA) levels, the testicular tissue was homogenized with 1.15% potassium chloride to obtain 1:10 (w/v) whole homogenate. The oxidative stress in testicular homogenate were assayed with the thiobarbituricacid reaction as formerlytestified and wasstated as nmolper gram tissue.The glutathione (GSH) content of testicular homogenates was determined at 412 nm according to the previously described method and statedas nmol per gram soft tissue.The superoxide dismutase (SOD) activity in thetesticular tissues was evaluated according to the method as described by Sun et al. (1988) wasused and was measured as U per gram protein.

For histopathological evaluation, the testes were sectionedin small pieces and were subjected to histopathological processingusing standard tissue processing protocolsas previously reported.[11, 14] Briefly, the tissues were dehydrated by using ordered ethanol solutions of strengths 50, 70, 80, and 90%, followed by two changes with 100%. The dehydrated tissues were then cleared in two changes each concentrated 100% xylene, after whichthey were penetrated and fixed in paraffin wax .Using rotatory microtome tissue were then cut at thickness of 4um. The tissue slides containing the sectioned paraffin tissue slices were stained with Harris hematoxylin and eosin (H & E).Each tissue slide was observed under a compound microscope attached to a digital camera. The photomicrographs were observed under an objective lens of 100x resolution. The tissues slides were assessed by an experiencedhistopathologist who was blinded to the various treatment groups.

Statistical analysis

The data were expressed as mean ± S.E.M and were analyzed using one-way ANOVA followed by Tukey's*post hoc*testusing GraphPad Prism 5 (GraphPad Software Inc. San Diego CA, USA). A P value of ≤ 0.05 was accepted as significant.

RESULTS

Effect of 6-hydroxyflavone on cisplatin-induced testicular oxidative stress

Administration of cisplatin at a single dose of 7.5 mg/kg was associated with a marked oxidative stress in the testicular tissue and produced a decrement in the tissues antioxidant markers.The production of oxidative stress was observed as a significant increase in the MDA level (P< 0.001) in the cisplatin vehicle-treated animals as compared to the group of animals administered only with the vehicle. The daily treatment with the flavonoid, 6-hydroxyflavone produced protection from the pathological influence of cisplatin testicular damage. When administrated at a dosage of 25 mg/kg, a substantial reduction in the levels of MDA (P< 0.01), GSH (P< 0.05) and SOD (P< 0.05) as compared to the cisplatin injection animals treated with the vehicle. An increase protection was afforded with 6-hydroxyflavone when the animals were treated with the higher dose of 50 mg/kg. This was noted as a substantialdiminution (P< 0.001) in the oxidative stress marker i.e. MDA level in the testicular tissues of animals injected with cisplatin. The enhancement in the antioxidant status was also significant at the higher dose of 6-hydroxyflavone (50 mg/kg) and was observed as a noteworthysurge (P< 0.001) in the content of GSH as well as a substantial increase (P< 0.001) in the activities of SOD, as associated to the vehicle treated cisplatin injected group of animals.Administration of the positive control, ascorbic acid at a dose of 50 mg/kg in the cisplatin injected animals was associated with a robust protective effect in the oxidative stressed testicular tissues as the reduction in the level of MDA level (P< 0.001) and the enhancement in the content of glutathione (P< 0.001) and activity of superoxide dismutase (P< 0.001) was significant, when compared to the group of animals treated with the vehicle and injected with cisplatin as shown in Table 1. No aberrant changes indicative of pathological changes in the testicular tissue was observed in the group of animals treated only with 6-hydroxyflavone at a higher dose of 50 mg/kg.

Values stated as mean ± SEM. $^{###}P < 0.001$ as compared to vehicle alone treated group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to cisplatin (Cis) un-treated group. One-way ANOVA followed by Tukey's *post hoc*test, $n = 6$ animals per group. Ascorbic acid (AA) was administered at 50 mg/kg (AA-50). 6-Hydroxyflavone (6HF) was administered at doses of 25 mg/kg (6HF-25)and 50 mg/kg (6HF-50).

Effect of 6-hydroxyflavone on cisplatin-induced histopathological changes in the testes

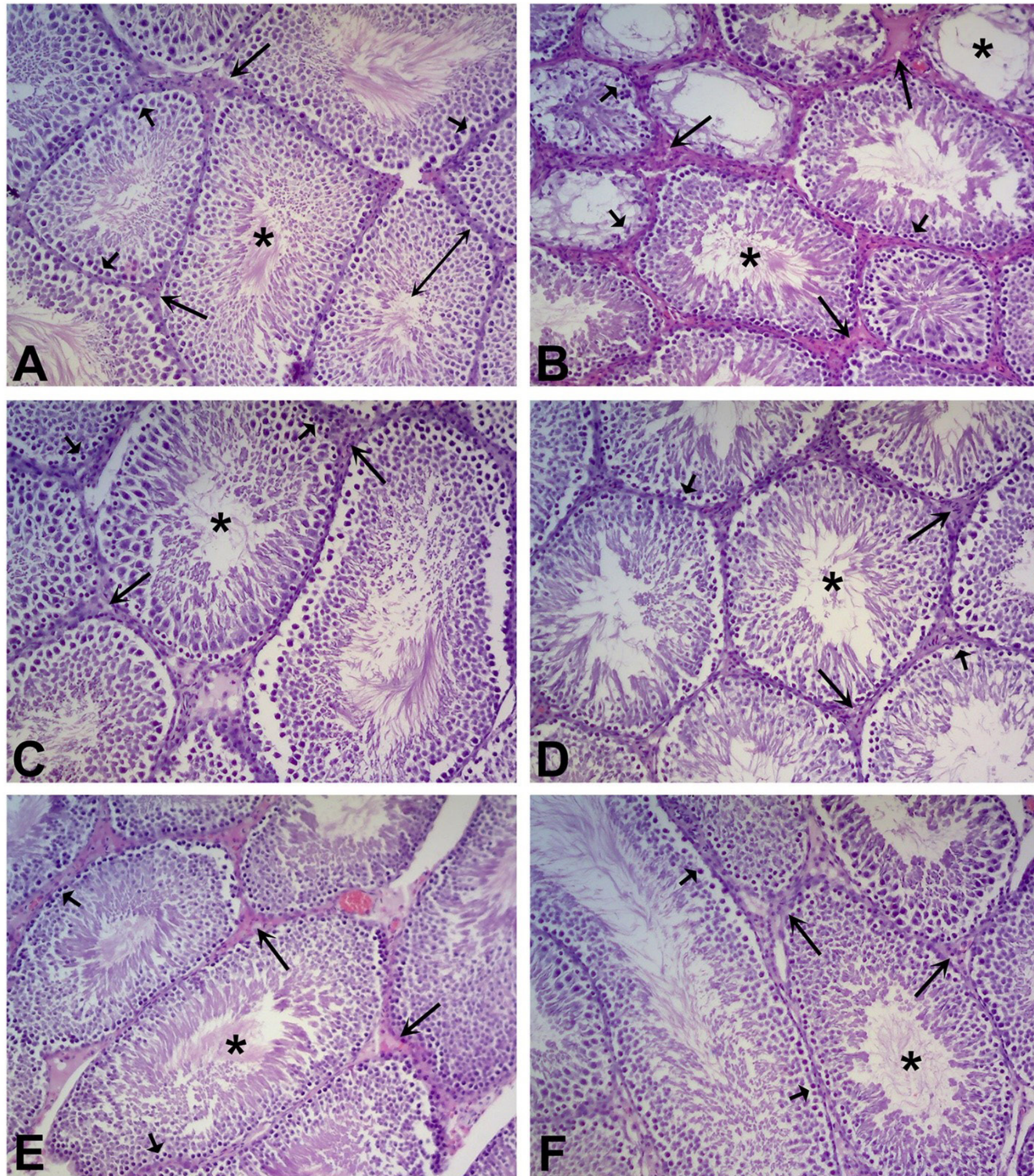

Figure 1: Histopathological evaluation of cisplatin-induced testicular toxicity after treatment with 6-hydroxyflavone and ascorbic acid (H & E; 100x original magnification). (A): Photomicrograph of a slice of testis from a rat treated with vehicle presenting normal appearing stratified germinal epithelium (double arrows line), consisting of Sertoli cells and spermatogenic cells with spermatogonia (small arrows) appear adjacent to the basement membrane and spermatids towards the adluminal compartment (asterisk) of the seminiferous tubule, which is bounded by an interstitial connective tissue (large arrows). (B): Photomicrograph of a segment of testis from a rat treated with cisplatin presentingshredding of the germinal epithelium in some seminiferous tubules, while other tubules showed severe necrotic changes (small arrows) with desquamated spermatids in the luminal compartment (asterisk) and thickening of the interstitial connective tissue (large arrows). (C): Photomicrograph of a slice of testis from a rat treated with 6-hydroxyflavone at 25 mg/kg showing almost normal histological features of the spermatogenic cells including spermatogonia (small arrows), spermatids towards the adluminal region (asterisk), and interstitial connective tissue (large arrows), except with mild shredding of the germinal epithelium. Normal histological features of seminiferous tubules containing a stratified germainla epithelium consisting of spermatogenic cells including spermatogonia (small arrows), and spermatids towards the lumen (asterisk) and surrounded by interstitial connective tissue (large arrows) are visible in the group of cisplatin injected animals treated with ascorbic acid at 50 mg/kg(D),6-hydroxyflavone at 50 mg/kg(E),and non-cisplatin injected animals treated with 6-hydroxyflavone at 50 mg/kg(F).

Table 1: Effect of 6-hydroxyflavone on cisplatin-induced testicular oxidative stress

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	SOD (U/g protein)
Vehicle	41.83±2.442	29.83±1.447	32.67±1.585
Cisplatin	102.0±4.058###	12.50±1.478###	11.67±1.145###
AA-50 + Cis	61.00±5.190***	21.67±1.022***	25.17±2.414***
6HF-25 + Cis	78.33±3.739**	19.17± 0.792*	21.17±2.358*
6HF-50 + Cis	67.67±5.308***	23.00±1.673***	26.00±1.862***
6HF-50	40.17±1.740	27.83±1.014	30.67±1.764

The single dose of cisplatin at 7.5 mg/kg produced significant degradable changes in the germinal epithelium of testes.In some of the seminiferous tubules, the pathological changes were observed as severe necrosis of the germinal epithelium. There was exfoliation and loosening of the germ cells with a reduction in the width of the germinal epithelium. These was increase in the width of the interstitial tissue around the seminiferous tubules with deposition of connective tissues. Degenerated spermatids appeared into the luminal compartment of the seminiferous tubules. There wasshredding of spermatogonia from the basement membrane of the seminiferous tubules. The spermatocytes and spermatids also appeared loosed from the germinal epithelium. Moreover, congestion and edematous changes were also observed in the interstitial tissue along with degeneration of Leydig cells. More detailed examination revealed cytoplasmic vacuolization, chromatolysis and diffusecavities in the germinal epithelium. Fusiform spermatocytes were also visible with degeneration of spermatogonia, reduction in the size of spermatocytes and disintegration of spermatids with necrotic changes. A decrease in the number of Sertoli cells with immature spermatozoeawas also visible in the seminiferous tubules. The group of animals treated with 6-hydroxyflavone at doses of 25 and 50 mg/kg showed almost normal histological features of the seminiferous tubules except minor shredding of the germinal epithelium was observed. Similarly, the positive control, ascorbic acid treated animals also presented with an almost normal histoaritecture of the germinal epithelium of the seminiferous tubules. No histopathological changes were detected in the sets of animals treated alone with the higher dose of 6-hydroxyflavone at 50 mg/kg (Figure 1).

DISCUSSION

The currentstudy evaluated the protective effect of the flavonoid, 6-hydroxyflavone against cisplatin associated testicular damage in rats.Platinum compounds significantly reduce the overall concentration of sperms, and produce sperm aneuploidy and alterations of DNA.[15] Cisplatin has been reported with long-term infertility including oligospermia or azoospermia.[16] Preclinical studies have shown that cisplatin severely damages the testicular tissue by causing apoptosis of germ cells, dysfunction of Leydig cells and testicular steroidogenic disorder. Cisplatinaffects spermatogenesis by inhibiting germ cells nucleic acid synthesis and produce damage to theLeydig cells thereby inhibiting the production of testosterone.[17]

In this study cisplatin produced severe degenerative changes in the germinal epithelium of the seminiferous tubules of testes. Cisplatin administration depletes germ cells, reduces the epithelium of the seminiferous tubules and producesfibrosis around the vessels in the interstitial spaces.[18] Thegerminal epithelium of the seminiferous tubules exhibits few spermatogonia and Sertoli cell. A reduction oftubular epithelial layers, having an irregular morphology containing few number of germ cells along with maturation arrest and perivascular fibrosis and intertubular tissue hyalinization have been observed after dosing with cisplatin.[19, 20] Almost similar cisplatin-induced histopathological changes in the testes have been observed in this study.

In this study, cisplatin was associated with a reduction of antioxidant and production of oxidative stress status in the testicular tissues. It has been reported that cisplatin-induced toxicity results from the production of free radicals in tissues.[21] Numerous studies have shown that exposure to cisplatin can disturb the antioxidant balance, and thus produceoxidative stress, thus leadingto various biochemical and physiological disturbances.[21, 22] Higher doses of cisplatin decrease body and organ weights and

increase the oxidative stress markers along with an increase in endoplasmic reticulum stress markers.[23] The sperms contain a unique lipid composition having high levels of polyunsaturated fatty acidsthat are responsible for the sperms flexibilities and motility, thus impacting their performance capabilities. The presence of lipids in the spermatozoa are the main precursors of lipid peroxidation, which may produce various abnormalities in the overall functions of sperms. Pathogenesis of testicular toxicityinduced after dosing with cisplatinandits associated infertility is associated with production of reactive oxygen species. Reactive oxygen species by damaging the sperm membrane and reduce its motility and ability to fuse with the ovum as well as also cause a direct damage to the sperm DNA, thus resulting in problems in the paternal genomic contribution to the embryo.[24-26]

In the present study, treatment with 6-hydroxyflavone protects the testes from the toxicological influence of cisplatin. Medicinal plants rich in flavonoids have shown beneficial effects in attenuating anti-cancer drugs induced toxicities of different organs.[27, 28] It has been reported that treatment with hesperidin at 50 mg/kg/day for 14 days reversed that cisplatin inflicted reduction in enzymatic and nonenzymatic antioxidants and oxidative stress as well as attenuates the cisplatin induced decrease motility of sperm, concentration of epididymal sperm, increase abnormal sperm rate and histopathological damage.[29] The plant, *Launeataraxacifolia* aqueous leaf at doses of 100 and 400 mg/kgfor 21 days lessened thecisplatin induced reduce sperm characteristics and increase sperm morphological abnormalities anddeformed histological picture of seminiferous tubules. The plant significantly increase the activities of superoxide dismutase and decreases lipid peroxidation , catalase and glutathione levels in the testes.[30] In an another study, treatment with naringenin-oxime prevents cisplatin-induced hepatotoxicity, nephrotoxicity, and genotoxicity by restoring the antioxidant system.[31] Treatment with the flavonoid,rutin results in retreatingthe cisplatin effect onsperm count, DNA damage, biochemicaland histological parameters.[32] Flavonoids are powerfulantioxidants effectthat hinder lipid peroxidation and sheltered the tissue from free radicals by uninterrupted scavenging reactive oxygen species, reactive nitrogen species, and activating antioxidant enzymes.

CONCLUSION

Cisplatin administration was associated with significant oxidative stress and severe histopathological changes in the testes. Daily treatment with the flavonoid, 6-hydroxyflavone at doses of 25 and 50 mg/kg reversed the cisplatin-induced testicular oxidative stress and preserved the germinal epithelium from the toxicological insult. These findings suggest that flavonoids may beused for effective management of anticancer drugs associated reproductive toxicity.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Khyber Medical College, Peshawar, Pakistan and University of Peshawar, Peshawar, Pakistanfor providing all the necessary facilities to carry out this research work.

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