Protective Effects of Blackberry Juice against Cisplatin-Induced Testicular Toxicity in Rats: Up-Regulation of Bcl-2 Proteins and Androgen Receptors

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ABSTRACT

Background: Testicular toxicity is one of the serious side effects of cisplatin, which is used in chemotherapy. Objectives: This study aimed to investigate the protective effects of blackberry juice on cisplatin-induced testicular toxicity in rats. Materials and Methods: A total of 32 male Wistar Albino rats were used in this study. The rats were divided into four groups including 8 rats in each. The first group (control group) received only water orally; the second group (BBJ group) was given BBJ 1.6 g/kg p.o.; the third group (CP group) received single dose CP 10 mg/kg i.p.; and the fourth group (CP + BBJ group) received single dose CP 10 mg/kg i.p. and BBJ 1.6 g/kg orally. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), levels in the testicular tissue and serum testosterone were biochemically evaluated. Testicular tissues histopathologically and immunohistochemically evaluated.

Results: CP caused an elevation in lipid peroxidation level (MDA) paralleled with significant decline in GSH content, SOD and CAT activities in testes as well as decrease of serum testosterone levels compared to control group. Some histopathological changes were detected in all testicular tissues of CP treated group in the form of degeneration and disorganization of germinal cells, irregular seminiferous tubules with decreased number of spermatogenic cells of seminiferous tubules. Co-administration of BBJ with CP ameliorates all the biochemical deteriorations with preserve the normal histological architecture of testes.

Conclusions: BBJ decreased CP induced testicular tissue damage as a potent free radical scavenger thanks to its antioxidant and anti-inflammatory properties. However, further experimental and/or clinical studies are required to confirm our results.

KEY WORDS

cisplatin, blackberry, oxidative stress, testicular toxicity, immunohistochemistry

INTRODUCTION

Testicular toxicity and infertility is a common problem associated with chemotherapy. Cisplatin (CP) used in treatment of different types of solid tumors is considered one of the most reported chemotherapeutic agents inducing this type of complication1. Cisplatin was reported to damage spermatogenesis as well as testosterone synthesis and one of the well-established mechanisms explaining cisplatin induced testicular toxicity is its ability to induce oxidative damage2. In experimental models, cisplatin was shown to damage spermatogenesis affecting sperm forms, count and motility in rats and oxidative stress parameters like increase testicular malondialdehyde as well as decreased testicular glutathione were noted3 and cisplatin administration as a chemotherapeutic agent4 is limited because its adverse effects, such as reproductive toxicity5. Moreover, it could increase the abnormality of spermatozoa and decrease the spermatozoa number and motility in patients suffering from neoplastic disorders. Steroidogenesis suppression and generation of free radicals are the main mechanisms of repro-
ductive toxicity which progress to testicular dysfunction. As a degenerative disorder, testicular dysfunction is characterized by failure in the synthesis of reproductive hormones and spermatogenesis. Several antioxidants have been tested previously to elaborate their potential to ameliorate cisplatin induced testicular toxicity including rutin, olive leaf extract, grape seed procyanidins extract and resveratrol. Their main mechanisms were ameliorating oxidative/nitrative stress and increasing expression of testosterone synthetase.

Blackberry (Rubus), belonging to the family Rosaceae, juice (BBJ) is rich with anthocyanins and polyphenols which are well known for their potent antioxidant effect. Further, lyophilized blackberries and blackberry extract were shown to have a potential in cancer chemoprevention with different mechanisms in rats as well as in human cells.

Studies on the use of BBJ in promoting the recovery of various degenerative disorders and its effects on the spermatogenesis of rats with cisplatin-induced testicular toxicity have not been conducted. Therefore, we aimed to investigate and explore the potential effect of BBJ on cisplatin-induced testicular dysfunction in rats as regards to oxidation, apoptosis and androgen receptor expression.

MATERIAL AND METHODS

Chemicals

Cisplatin, in the form of (CP 10 mg/ vial), was purchased from Mylan Institutional LLC, Rockford, IL USA. Fresh black berry fruits (Rubus spp.) were purchased from the local market (Ismailia, Egypt). The fruits were washed, homogenized and their juice was freshly prepared daily. Black berry dose (1.6 g/kg bw equal to 9 ml/kg bw) was selected based on earlier studies. All kits were purchased from a Bio-diagnostic company for diagnostic and research reagents (Dokki, Giza, Egypt) and Sigma-Aldrich Chemical Co. (USA). All other chemicals and solvents were of analytical grade.

 Experimental animals

In this experimental study, a total of thirty-two of SPF (Specific Pathogen Free) healthy sexually mature adult male Wistar rats (2 months old, 150-200 g) were used. The animals were obtained from Faculty of Veterinary medicine, Suez Canal University and used in this study. They were maintained on standard pellet diet and tap water ad libitum and were kept in small cages (8 animals per each) with wood chip bedding under a 12-h light/dark cycle and room temperature 22-24°C. Rats were acclimatized to the environment for 2 weeks prior to experimental use. All experimental protocols follow the guidelines for animal care and use in experimentation of the Bioethical research Committee of Faculty of Medicine, Suez Canal University and Helsinki Declaration in 1975 (revised in 2008).

 Experimental protocol

Rats were randomly distributed into four groups; eight rats each. Groups were treated as follows:

- Control group (G1): received water by oral gavage (5 ml kg⁻¹ body weight) for 21 days and received one intraperitoneal injection (i.p.) of saline on the 16th day (5 ml/kg body weight).
- Blackberry juice group (G2) received BBJ (1.6g kg⁻¹; p.o.) for 21 days.
- CP group (G3) was given CP in a single dose (10 mg kg⁻¹; i.p.) on the 16th day of treatment, a dose that induced testicular toxicity in rats.
- (CP + BBJ groups) (G3) Rats of the protective groups received BBJ for 21 days, 1 h after a single dose of CP on the 16th day of treatment. BBJ was administrated orally at the same dose as group 2. Doses of BBJ were selected based on previously reported pharmacological properties of these agents.

 Body and reproductive organ weights

Initial and final body weights were recorded at the beginning and end of the experiment, respectively. All rats were sacrificed, after anesthesia, by cervical dislocation. Testes were extracted, blotted and weighed. Gross body and reproductive organs signs were examined for toxicological parameters. The relative testis weight to body weight was calculated as the ratio of tissue wet weight (g) to body weight (g) multiplied by 100.

Collection of Blood and Testis Specimens

At the end of treatments, animals were weighed and sacrificed. The testes were excised, weighed, a part of each testis tissue was collected and fixed in Bouin's solution then processed and embedded in paraffin for histopathological examination, and other parts were kept in -80°C and thawed just before homogenization in phosphate buffered saline for the biochemical assay.

Hormonal Assay

All blood samples were obtained during morning hours with respect to the diurnal variability of testosterone hormone. Samples were centrifuged for 15 min at 3000 rpm using a bench centrifuge, and plasma was stored at 4°C for testosterone (ng/mL) assay using a commercially available ELISA kits in accordance with the manufacturer's instructions.

Biochemical assessment of oxidative stress

Malondialdehyde (MDA) measurement

Testicular malondialdehyde (MDA) level was detected biochemically. Trichloroacetic acid was added to the sample for protein precipitation and then thiobarbituric acid was added. The mixture was heated for 10 min in a boiling water bath. One molecule of MDA in the homogenized testis samples reacted with two molecules of thiobarbituric acid and the resulting chromogen was centrifuged. The intensity of the color developed in the supernatant was measured spectrophotometrically at 535 nm.

Reduced glutathione (GSH) measurement

Reduced glutathione (GSH) level was estimated following the method described by Ellman. In this method thiols react with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB), cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (TNB-), which ionizes to the TNB- dianion in water; 15 μL of sample was mixed with 260 μL assay buffer (0.1 M sodium phosphate and 1 mM EDTA, pH 8) as well as 5 μL Ellman reagents; incubated for 15 min at room temperature and the TNB- formation was quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Catalase activity measurement

Catalase activity was determined spectrophotometrically by the method of method described by Ellman. In this method thiols react with Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB), cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (TNB-), which ionizes to the TNB- dianion in water; 15 μL of sample was mixed with 260 μL assay buffer (0.1 M sodium phosphate and 1 mM EDTA, pH 8) as well as 5 μL Ellman reagents; incubated for 15 min at room temperature and the TNB- formation was quantified in a spectrophotometer by measuring the absorbance of visible light at 412 nm. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Superoxide dismutase (SOD) measurement

Testicular superoxide dismutase (SOD) activity was detected biochemically. The method used to determine SOD activity in homogenized testis samples is based on the autoxidation of pyrogallol which is inhibited by SOD. One unit of SOD is generally defined as the amount of enzyme that inhibits the autoxidation of pyrogallol by 50%. The activity of SOD was monitored spectrophotometrically at 420 nm.

Histopathology

The fixed testis tissue in Bouin’s solution for 48 h slices were processed for paraffin embedding. Two nonconsecutive 4-μm thick sections per animal were prepared. Subsequently, the testicular sections stained with hematoxylin and eosin (H&E) were histopathologically examined. Histopathologic examination of slides from each specimen was done on
Table 1. Changes in body weight and testis weight of rats after exposure to cisplatin, Blackberry juice and their combination. n = 8 rats for each group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>G1 (Control)</th>
<th>G2 (Blackberry juice)</th>
<th>G3 (Cisplatin)</th>
<th>G4 (cisplatin+ Blackberry Juice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBW (g)</td>
<td>191.55 ± 5.19</td>
<td>193.05 ± 4.15</td>
<td>194.22 ± 3.36</td>
<td>196.33 ± 5.13</td>
</tr>
<tr>
<td>FBW (g)</td>
<td>240 ± 8.76</td>
<td>239.33 ± 6.50</td>
<td>207.83 ± 4.26</td>
<td>231.50 ± 6.92</td>
</tr>
<tr>
<td>RBW</td>
<td>125.34 ± 4.64</td>
<td>123.97 ± 1.66</td>
<td>107.03 ± 2.66</td>
<td>117.99 ± 4.92</td>
</tr>
<tr>
<td>TW</td>
<td>1.40 ± 0.02</td>
<td>1.37 ± 0.02</td>
<td>1.07 ± 0.03</td>
<td>1.31 ± 0.06</td>
</tr>
<tr>
<td>RTW</td>
<td>0.58 ± 0.02</td>
<td>0.57 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

Data are mean ± SD. G1: Control; G2: Blackberry Juice; G3: Cisplatin; G4: Cisplatin + Blackberry Juice.


Relative body weight = (final body weight / initial body weight) x 100; Relative testis weight = (testis weight / final body weight) x 100

a: compared to the control (G1) group  b: compared to Blackberry juice (G2) group.

c: compared to Cisplatin (G3) group - Significant at p < 0.05

Table 2. Changes in serum levels of testosterone and oxidative stress markers in the testis of rats after exposure to cisplatin, Blackberry juice and their combination. n = 8 rats for each group.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>G1 (Control)</th>
<th>G2 (Blackberry juice)</th>
<th>G3 (Cisplatin)</th>
<th>G4 (cisplatin+ Blackberry Juice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (ng/ml)</td>
<td>0.90 ± 0.02</td>
<td>0.89 ± 0.03</td>
<td>0.24 ± 0.03</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>CAT (U/mg tissue)</td>
<td>32.67 ± 2.50</td>
<td>28.17 ± 2.93</td>
<td>16.00 ± 1.26</td>
<td>26.67 ± 2.16</td>
</tr>
<tr>
<td>SOD (U/mg tissue)</td>
<td>31.00 ± 2.37</td>
<td>29.67 ± 3.20</td>
<td>16.83 ± 2.32</td>
<td>28.50 ± 3.94</td>
</tr>
<tr>
<td>GSH (mg/gm tissue)</td>
<td>86.90 ± 2.04</td>
<td>85.78 ± 3.20</td>
<td>44.43 ± 3.80</td>
<td>73.65 ± 5.48</td>
</tr>
<tr>
<td>MDA (mmol/g tissue)</td>
<td>33.20 ± 2.38</td>
<td>34.13 ± 2.52</td>
<td>75.30 ± 4.72</td>
<td>50.18 ± 5.10</td>
</tr>
</tbody>
</table>

Data are mean ± SD. G1: Control; G2: Blackberry Juice; G3: Cisplatin; G4: Cisplatin + Blackberry Juice.

CAT: Catalase; SOD: Superoxide dismutase; GSH: Glutathione; MDA: Malondialdehyde; TS: testosterone.

a: compared to the control (G1) group  b: compared to Blackberry juice (G2) group.

c: compared to Cisplatin (G3) group - Significant at p < 0.05.

Effect of CP and BBJ on body weight and Testes Weights

As shown in Table 1, cisplatin has significantly decreased final body weight, relative body weight, testis weight and relative testis weight as compared to control groups (p < 0.05). Co-administration of BBJ with CP has significantly reversed all these parameters when compared with CP alone (p < 0.05).

Effect of BBJ on Cisplatin-induced oxidative stress markers and serum testosterone levels

As illustrated in Table 2, animals received cisplatin showed a marked depletion in the testicular GSH content, catalase, superoxide dismutase activities as well as declined serum testosterone level and it remarkably initiated the oxidative stress as indexed by increased level of MDA as compared to normal control rats (p < 0.05). Co-administration of BBJ with CP provided moderate normalization of serum testosterone concentration in group 4 compared to cisplatin group (G3) with significant improvement in the antioxidant parameters (p < 0.05), as well as significant reduction of MDA levels in group 4 compared to CP group (p < 0.05).

Effect of BBJ on Histopathological Changes after Cisplatin Administration

Rats in the control group showed normal testicular architecture with normal arrangement of the germinal cells, regular course of spermatogenesis, Sertoli cells, and Leydig cells without histopathological lesions (Fig. 1A). Animals treated with BBJ were characterized by normal appearance as control group with regular seminiferous tubules with normal stratified epithelium, spermatogenic cells and Leydig cells (Fig. 1B). All rats treated with cisplatin showed degeneration and disorganization of germinal cells, irregular seminiferous tubules with decreased number of spermatogenic cells of seminiferous tubules. Some pyknotic cells and apoptotic Leydig cells appeared. All stratified spermatogenic cells are decreases in number and size (Fig. 1C). Regular seminiferous tubules with Sertoli cells with some spermatogenic cells of seminiferous tubules starts to be stratified epithelium in CP + BBJ treated group (Fig. 1D).

AR and Bcl-2 protein expression

In testicular cells of control rats and BBJ group, weak immunoreactivity against AR was detected as shown in Fig. 2A & 2B. AR positive staining was observed as coarse brown granules in the cytoplasm in spermatogenic cells of rats at CP treated groups (Fig. 2C). Bcl-2 immu-
is often advised to patients before receiving chemotherapy. Our aim was to assess the potential protective role and the mechanism of action of BBJ on cisplatin induced testicular toxicity and infertility. Thus, serum banking of many types of malignancies yet causing some important adverse effects on the testicular tissue of rats.

BBJ has not been evaluated before to ameliorate cisplatin induced adverse effect of cisplatin on testicular tissues. To the best of our knowledge, we demonstrated that co-administration of BBJ can ameliorate the harmful effects of structural and functional deterioration induced by cisplatin.

In our study, cisplatin has been shown to damage the reproductive organs of rats and cause loss of spermatogenic cells compared to the control group (Fig. 3A & 3B). In rats exposed to CP, Bcl-2 protein particles were unevenly distributed and the number of positively-stained cells decreased when compared with the control group (Fig. 3C).

**DISCUSSION**

Cisplatin is a widely used chemotherapeutic agent used in treatment of many types of malignancies yet causing some important adverse effects including testicular toxicity and infertility. Thus, serum banking is often advised to patients before receiving chemotherapy. Our aim was to assess the potential protective role and the mechanism of action of BBJ on cisplatin induced testicular damage in rats. Our results demonstrated that co-administration of BBJ can ameliorate the harmful effect of cisplatin on testicular tissues. To the best of our knowledge, BBJ has not been evaluated before to ameliorate cisplatin induced adverse effects on the testicular tissue of rats.

CP has significant decrease in the physical weight and reproductive organ weights. It has been reported that administration of anticancer drugs to the male rats decreases reproductive organ weights. In the current study, administration of CP reduced testes weights after treatment when compared with control, confirming previous reports that CP decreases reproductive organs weights. Reductions in testes weights are attributed to parenchymal atrophy in the seminiferous tubule (ST) of rats after CP administration. BBJ partially attenuated the reduction in the weights of testes after CP treatment. The weight of testes is largely dependent on the mass of differentiated spermatogenic cells. The reduced tubule size, decreased number of germ cells and elongated spermatids may lead to the reduction in the weight of testes as observed in this study.

In our study, cisplatin has been shown to damage the reproductive capabilities of rats as evident both histologically and biochemically. It affected the seminiferous tubules, significantly decreased serum testosterone and significantly deteriorated oxidative stress parameters namely testis MDA, catalase, superoxide dismutase and reduced glutathione. Similar findings were previously noted and cisplatin has been also shown to possess deleterious effect on sperm count, motility and spermatogenic cell density.

In the present study, catalase (CAT) and superoxide dismutase (SOD) levels were significantly declined in CP treated groups as compared to control animals. These findings are in accordance with previously reported studies in which CP treatment caused reduction in CAT levels. Increased levels of ROS and RNS will lead to lipid peroxidation and oxidative stress that can lead to cell damage which is irreversable.

Parenchymal atrophy in the seminiferous tubules had been reported after the administration of cisplatin, which causes loss of spermatogenesis, abnormalities in the Leydig cells, and suppression of testosterone production. Cisplatin-induced testicular damage was characterized by cell apoptosis, Leydig cell dysfunction, and infertility.

These histopathological findings in our study explained the inhibition of testosterone production and spermatogenesis. Previous authors reported that CP-induced testicular cell damage is caused by oxidative stress, which induces cellular damage by the generation of reactive oxygen species. DNA damage, cell replication and transcription interference are caused by oxidative stress. This pathogenesis of structural and functional deterioration could explain the process of cell damage induced by cisplatin.

In the current study, CP also resulted in a decrease in serum testosterone levels, while CP + BBJ group had significantly higher levels. In another study, testosterone levels measured following the administration of CP, the authors found decreased testosterone levels in the CP group, compared to the control group. Total testosterone level was also increased in the recovery group where BBJ is co-administered with CP. CP cause damage of the Leydig cells, so the testosterone production is hampered. This improvement may be to BBJ antioxidant and free radical scavenger activities.

BBJ has been shown to protect the testes from the damage produced by cisplatin. One of the mechanisms was via amelioration of the oxidative damage produced by cisplatin as evident by BBJ ability to significantly increase the testicular catalase, superoxide dismutase and reduced glutathione levels. BBJ has also significantly reduced serum MDA levels. These results come in agreement with previous studies of blackberry.
ry juice and extract in other models which have demonstrated the ability of blackberry to significantly recover the activity of the antioxidant enzymes and to successfully oppose in vivo oxidative stress[8,30].

-added to BBJ ability to significantly increase serum testosterone levels, there's another protective effect that was noticed in our study; the ability of BBJ to significantly up-regulate androgen receptor expression in testes of rats exposed to cisplatin. To the best of our knowledge, this study is the first one to show such a positive finding, however, these results resemble those of the Korean herbal formula (Modified Ojayeongjanghwon) in another rat model 34).

This study demonstrates a protective effect exerted by BBJ against cisplatin-induced testicular toxicity in rats. This effect was shown to be exerted, at least partly, by BBJ antioxidant, antiapoptotic effects as well as by the ability to up-regulate androgen receptor expression. This BBJ potential encourages performing other experimental as well as clinical studies to elucidate more mechanisms as well as to test its clinical protective potential as regards to chemotherapy induced testicular toxicity.

**CONCLUSION**

REFERENCES


