**Original paper**

Anti-apoptotic Effect of Grape Seed Proanthocyanidin Extract on Cisplatin-induced Apoptosis in Rat Testis

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Received January 9, 2015; Accepted May 19, 2015

This study was to investigate the possible anti-apoptotic effect of grape seed proanthocyanidin extract (GSPE) on cisplatin (CDDP)-induced testicular apoptosis in male rats. The control group was treated with physiological saline, the GSPE (400 mg/kg) group, GSPE (200 mg/kg) + CDDP group and GSPE (400 mg/kg) + CDDP group were received GSPE orally at corresponding dose respectively, for 15 consecutive days, starting 10 days before a single intraperitoneal dose of CDDP (7 mg/kg) in CDDP group and GSPE + CDDP groups. Administration of CDDP significantly reduced the weight of testis and epididymis, sperm concentration and caused significantly increase in the apoptosis rate of testicular cells and expression of Bax and Caspase-3, decrease in the expression of Bcl-2, along with some histopathological lesions in testicular tissue. Therefore, pre-treatment of GSPE to CDDP-injected rats tend to prevent CDDP-induced lose weight of organs, testicular apoptosis and histopathological lesions. Presented data have documented GSPE have improvement effects against testicular apoptosis caused by CDDP.

Keywords: cisplatin, grape seed proanthocyanidins, testis, Apoptosis

**Introduction**

Cisplatin (cis-diaminedichloroplatinum(II), CDDP) is a widely prescribed antineoplastic DNA alkylating agent used to treat many types of cancer such as testis, ovarian, bladder and lung. Despite the significant therapeutic effect of CDDP, various adverse effects, such as nephrotoxicity, reproductive toxicity and ototoxicity, limit its clinical application. Impairment of reproductive organs, especially testicular damage, has gained increasing attention in recent years, owing to the high incidence during or after the chemotherapy with CDDP (Atessahin et al., 2006). However, the cellular mechanisms of testicular injury caused by cisplatin were poorly understood. Several studies have shown that pathogenesis of renal, hepatic, testicular damage following CDDP exposure is generally ascribed to oxidative damage (Chirino, Pedraza-Chaverri, 2009; Gaona-Gaona et al., 2011; Ahmed et al., 2011), in addition, an increase in apoptotic rate of germ cell is seen because of the side effects of cisplatin (Amin et al., 2008; Lirdi et al., 2008).

Grape seed proanthocyanidin extract (GSPE), are naturally occurring polyphenolic compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark. GSPE has been shown to serve as one of the most potent free radical scavengers and antioxidants both in vitro and in vivo. Oligomeric proanthocyanidins was provided to be highly bioavailable and provide significantly greater protection against damage of oxidative stress than vitamins C, E and β-carotene (Zhang et al., 2005; Saad et al., 2009). Increased interest in proanthocyanidins is based on the fact that they are believed to be non toxic, bioavailable and provide significant multi organ protection against drug and chemical induced toxic assaults (Yousef et al., 2009).

Therefore, the present study was designed to investigate whether GSPE has a anti-apoptotic effect against CDDP-induced negative changes in apoptosis rate of testicular cells, expression of...
Materials and Methods

Chemicals  CDDP (freeze-dried powder for injection) was obtained from Shandong Qilu Pharmaceutical Factory.

GSPE (purity is more than 95% as analysed by UV, in which dimer is 56%, trimer is 12%, tetramer is 6.6% and Monomer and other high-molecular mass oligomers is 20.4% as analysed by HPLC) was purchased from Tianjin Peak Natural Product Research Development Co., Ltd.

Primary antibodies: anti-rabbit caspase-3 polyclonal antibody (NO.SC-7148, detection of caspase-3 p11, p17 and p20 subunits and full length procaspase-3), anti-rabbit Bcl-2 polyclonal antibody (NO.SC-492), anti-rat Bax monoclonal antibody (NO.SC-7480) were purchased from Santa Cruz-Chinese Sequoia Jinqiao Biotech Corp.

Animals and Treatment Design  This study was conducted in accordance with our institutional guidelines on the use of live animals for research, and the experimental protocol was approved by the Experimental Animal Ethical Committee of Function Test Center for Functional Food, College of Arts and Science, Beijing Union University. Fifty adult male Sprague-Dawley rats (140 – 160 g) were purchased from the Laboratory of Animal Center of Academy of Military Medical Sciences of China, and were housed under standard laboratory conditions (12h light, 12h dark and 24 ± 3°C).

The rats were randomly divided into five groups as follows: (1) the control group, given sterile water (0.5 mL/100 g. bw) by gavage for continuous 15 days. (2) the CDDP group, given sterile water (0.5 mL/100 g. bw) by gavage for continuous 15 days, starting 10 days before a single intraperitoneal dose of CDDP (7 mg/kg), the dose is well known to induce testicular toxicity in rats (Libey et al., 2009). (3) the GSPE group, given GSPE (400 mg/kg) which was suspended in distilled water by gavage for continuous 15 days. (4) the CDDP + GSPE (200 mg/kg) group, given GSPE (200 mg/kg) solution by gavage for continuous 15 days, starting 10 days before a single intraperitoneal dose of CDDP (7 mg/kg). (5) the CDDP + GSPE (400 mg/kg) group, given GSPE (400 mg/kg) solution by gavage for continuous 15 days, starting 10 days before a single intraperitoneal dose of CDDP (7 mg/kg).

Sample Collection  The rats in all groups were sacrificed on the fifth day after CDDP injection. The testis, epididymis were removed, cleared of adhering connective tissue. After weighting the testis and epididymis, one of the testis was fixed in 10% formalin for histopathological examinations, a part of the other testis was fixed in 70% ethanol for apoptosis detection, and the rest of the testis was weighted, then added culture medium (DMEM) into it at concentration of 1 mg/mL for sperm count.

Sperm Concentration  Spermatozoa in the testis or epididymis were counted by a modified method of Yokoi et al (Yokoi, 2003). Briefly, the testis or epididymis were minced with anatomical scissors in 5 mL of physiological saline, placed in a rocker for 10 min, and incubated at room temperature for 2 min. The supernatant fluid was diluted as 1:100 with a solution containing 5 g of sodium bicarbonate, 1 m formalin (35%) and 25 mg eosin per 100 mL of distilled water. Total sperm number was determined with a hemocytometer. Approximately 10 dl of the diluted sperm suspension was transferred to each counting chamber and was allowed to stand for 5 min for counting under a light microscope at 200× magnification.

Histopathological Examination  The testicle samples that fixed in 10% formalin were processed (dehydrated in graded concentrations of alcohol, immersed in xylene) and embedded in paraffin. Sections were cut at 5μm thicknesses on a rotary microtome, mounted and then stained with hematoxylin and eosin (H&E). The sections were evaluated under a light microscopy.

Measurement of Apoptotic Cells by Flow Cytometry

a) The Preparation of Single Cell Suspension
The testicle samples that fixed in 70% ethanol were cut into pieces and grinded gently on the cellular grid and mixed with saline solution to form the cell suspension, in which cell concentration was about 1×10⁶/mL.

b) Cell Apoptosis Detection
The cell suspension was subjected to propidium iodide (PI) staining in binding buffer at 4°C for 30 min in the dark. The stained cells were analyzed by flow cytometry (Epics-XLII Beckman Coulter, US). The percentage of cells in sub-G1 phase denote the apoptosis rate.

The flow cytometry was employed with the 15 mW ion laser as emission resource, and the wavelength of emission light was 488 nm. The Immunofluorescence data was analysed by Expo 32 ADC software, and the cell cycle of DNA was analysed by Muticycle AV software. The variation coefficient (CV) of the machine was adjusted as < 2% using flow-check Fluospheres (10 μm) Fluorescent Microspheres (REF 6605359. Beckan Coulter, Inc. Fullerton, CA 92835).

c) Detection of Caspese-3, Bcl-2, Bax Expression
100 μL anti-rabbit caspase-3 polyclonal antibody (1:100) or anti-rabbit Bcl-2 polyclonal antibody (1:100) or anti-rat Bax monoclonal antibody (1:100) was added into the tube containing 100 μL cell suspension and mixed sufficiently. After being incubated at room temperature for 30 min, the antibody-incubated cells were washed by 10 mL PBS solution, then 100μL goat anti mouse FITC-IgG antibody (1:50) was added into the tube. After being incubated at room temperature for 30 min in dark, the antibody-incubated cells were washed again as above. After centrifuged at 32×g for 3 – 5 min, the supernatant of the sample solution was decanted. Finally 100 μL PBS were used to resuspend the precipitate. Flow cytometry was used to analyze the expression of caspase-3, Bcl-2 and Bax. The parameters of the flow cytometry instrument Settings and data analysis software are the same as the description in Measurement of Apoptotic Cells by Flow Cytometry.
Statistical Analyses Results of all groups were shown as means ± SD and analysed by SPSS program (version 12.0). Values were compared by one-way analysis of variance (ANOVA) and post hoc Duncan (D) test to determine the differences among all the groups. P < 0.05 was accepted as a statistically significant value.

Result

Organ Weights As shown in Fig. 1, a significant decreases in weights of testis (P < 0.01) and epididymides (P < 0.01) were observed as a result of CDDP administration as compared to the control group. Pre-treatment of GSPE (400 mg/kg) significantly improved CDDP-induced changes (P < 0.01). GSPE (200 mg/kg) also increase the weights of testes and epididymides, but no significance appeared.

Sperm Concentration Testicular sperm concentration and epididymal sperm concentration are shown in Fig. 2. Administration of CDDP significantly decreased sperm concentration (P < 0.01) compared with the control group, whereas pre-treatment with GSPE (400 mg/kg) significantly prevented the CDDP-induced side effects in sperm concentration compared with CDDP group (P < 0.01). However, GSPE (200 mg/kg) non-significantly increased the sperm concentration compared with CDDP group.

Histopathological Observations Degeneration, necrosis and interstitial oedema were detected in testicular structure of CDDP group (Fig.3B) compared with the control group (Fig.3A). Pre-treatment of GSPE (200 mg/kg) to CDDP-treated rats improved the structure damage slightly (Fig.3D), while the administration of GSPE (400 mg/kg) to CDDP-treated rats improved nearly all the CDDP-induced damages in the structure of testis (Fig. 3E). However, treatment with only GSPE had no effect on the testicular structure compare with the control group (Fig. 3C).

Cell Apoptosis Rate As shown in Table 1 and Fig 4, administration of CDDP significantly increased the rate of testicular apoptosis in rats while treatment of only GSPE had no influence on apoptotic rate. However, compared with CDDP group, pre-treatment with GSPE (400 mg/kg) before CDDP injection significantly (P < 0.05) improve CDDP-induced the increase in testes apoptotic rate.

Changes of Apoptosis-related Protein Expression The expression level of caspase-3, Bcl-2 and Bax in testis are shown in Table 2. The level of caspase-3 (P < 0.01) and Bax (P < 0.05) were increased significantly in CDDP group compared with that in control group, while the level of Bcl-2 (P < 0.01) was decreased significantly in CDDP group compared with that in the control group. However, administration of GSPE (400 mg/kg) significantly prevented the level of Caspase-3 (P < 0.01) and Bax (P < 0.05) from increasing, as well as the level of Bcl-2 (P < 0.05) from decreasing. The ratio of Bcl-2 to Bax (Bcl-2/Bax) was calculated as an index of apoptotic signaling. The Bcl-2/Bax ratio (P < 0.05)
was decreased significantly in CDDP group compared with control group, pre-treatment with GSPE can increase the Bcl-2/Bax ratio, but it have no significant differences among the CDDP group and GSPE+CDDP groups.

**Discussion**

Cisplatin is an effective chemotherapeutic, however the anticancer therapy is limited by its adverse reactions that have been documented in various experimental studies (Anderson et al., 1995; Kaur et al., 1997; Cherry et al., 2004). Testicular dysfunction is one of the most common long-term side effects of this therapy (Howell, Shalet, 2001). In recent years, the protection of testicular toxicity has get increasing attention. In our previous studies (Zhao et al., 2014), the protective role of GSPE against CDDP-induced oxidative stress effect has been demonstrated. In this study, for further research of the mechanism in CDDP caused testicular

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**Fig. 3.** Effect of GSPE on testis histopathology in CDDP induced rat cisplatin nephrotoxicity. (A) control group (H&E, 200×). (B) CDDP group (H&E, 200×). (C) GSPE (400 mg/kg) group (H&E, 200×). (D) CDDP + GSPE (200 mg/kg) group (H&E, 200×). (E) CDDP + GSPE (400 mg/kg) group (H&E, 200×).
toxicity, we investigated whether GSPE have protective effects on CDDP-induced testicular cell apoptosis.

Some investigators (Giri et al., 1998; Cherry et al., 2004; Colpi et al., 2004; Howell, Shalet, 2001) have reported that CDDP administration caused temporary or permanent azoospermia or oligospermia. In the present study, administration of CDDP reduced testicular and epididymal weights, sperm concentration when compared with the control group, indicating that CDDP caused testicular damage. In accordance with the reduction of organ weights and sperm concentration, histopathologic

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**Table 2.** Changes in the expression of apoptosis-related proteins in rat testicular cells in response to treatment of CDDP and GSPE. (means ± SD, n = 10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Caspase-3</th>
<th>Bcl-2</th>
<th>Bax</th>
<th>Bcl-2/Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>193.01±16.53</td>
<td>250.60±13.41</td>
<td>299.97±20.39</td>
<td>0.95±0.12</td>
</tr>
<tr>
<td>CDDP</td>
<td>254.17±15.46 (^a)</td>
<td>219.43±21.80 (^a)</td>
<td>327.14±31.39 (^a)</td>
<td>0.82±0.08 (^a)</td>
</tr>
<tr>
<td>GSPE</td>
<td>181.84±26.70</td>
<td>265.12±17.55</td>
<td>288.74±20.86</td>
<td>0.96±0.06</td>
</tr>
<tr>
<td>CDDP+GSPE (200mg/kg)</td>
<td>211.55±15.24 (^d)</td>
<td>239.10±18.43</td>
<td>318.29±16.68</td>
<td>0.84±0.06</td>
</tr>
<tr>
<td>CDDP+GSPE (400mg/kg)</td>
<td>202.10±16.52 (^d)</td>
<td>249.34±25.63 (^c)</td>
<td>297.43±5.66 (^c)</td>
<td>0.90±0.09</td>
</tr>
</tbody>
</table>

**Note.** \(^a\)P < 0.05, \(^b\)P < 0.01 compared with control group. \(^c\)P < 0.05, \(^d\)P < 0.01 compared with CDDP group.
examination showed severe degeneration, necrosis in the testis of rats treated with CDDP alone, reflecting the testicular damage directly. Our findings are compatible with report of some researchers (Türk et al., 2011) and confirm the sperm toxic effects of CDDP in rats.

It has been known that exposure to CDDP (Türk et al., 2011) causes the rise of germ cell apoptotic rates. In the present study, intraperitoneal injection of CDDP significantly increased the apoptosis rate of testicular seminiferous tubule cell. The intrinsic pathway of apoptosis is critically regulated by pro-apoptotic and anti-apoptotic members of the Bcl-2 family. Bcl-2 and several close relatives inhibit apoptosis, whereas structurally similar relatives such as Bax and distant cousins such as Bik and Bim promote apoptosis. The proteins of Bcl-2 and Bax coexist dynamically inside cells, reflecting the dynamical relationship between the expressions of the two genes. The dynamical relationship can be indicated by the value of Bcl-2 / Bax ratio. Bcl-2 and Bax can form into heterologous dimers in the condition of Bcl-2 over-expression. Whereas, homologous dimers will be formed by Bax and Bax in the condition of Bax over-expression (Wang, Zheng, 2004). Meanwhile, Caspases are cysteine proteases that play a central role in the execution of apoptosis (Enoksson, 2004).

There are at least two major apoptotic pathways, initiated by caspase-8 and caspase-9 respectively, which can activate caspase cascades, the key link of which is activation of executioners caspase-3 (An, 2004). It has been reported that Bcl-2, anti-apoptotic protein localized in mitochondria, can be cleaved by caspase-3 and thus be converted to a proapoptotic protein similar to Bax (Kirsch et al., 1999; Carvalho et al., 2013). The oligomerization of Bax in the mitochondrial membrane has been shown to induce cytochrom c release and the subsequent steps (including caspase-9 and caspase-3) in the execution phase of apoptosis (Punn et al., 2000; Quadri et al., 2005). In present study, only treatment with CDDP significantly down-regulated the expression of Bax and caspase-3, while up-regulated the expression of Bcl-2, indicating that testicular apoptosis possibly due to changes in expression level of Bax, caspase-3 and Bcl-2 caused by cisplatin.

Proanthocyanidins have also been shown to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and to affect enzyme systems including phospholipase A2, cyclooxygenase, and lipooxygenase. Furthermore, proanthocyanidins was proved to have the ability to inhibit the activity of xanthine oxidase, a major generator of free radicals (Fine, 2000). A large number of reports demonstrated that oligomeric proanthocyanidins (OPCs) could both enhance the activity of chemotherapeutic agents and diminish their normal tissue toxicity (Yamakoshi et al., 2002). In this study, increase in weights and sperm concentration of testis and epididymis were observed in CDDP + GSPE (400 mg/kg) group compared with that in the CDDP group. In addition, pre-treatment of GSPE before CDDP injection lowered the sperm apoptotic rate than that only treated with CDDP, which may be owing to the up-regulated expression of Bcl-2 and down-regulated expressions of Bax and caspase-3 induced by GSPE as shown in the results.

In conclusion, this study apparently suggests that GSPE have improvement effects against testicular apoptosis induced by CDDP. These improvement effects of GSPE seem to involve regulation in expressions of apoptotic relative genes, such as Bax, Bcl-2, and caspase-3. Therefore, GSPE may be used in combination with CDDP in cancer patients to improve CDDP-induced germ cell apoptosis.

Acknowledgements This work was supported by grants from Scientific Research Project of Beijing Union University: Opening Project of Key Laboratory (NO.Zk70201502).

References
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