Epigallocatechin-3-gallate counters cisplatin toxicity of rat testes

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ABSTRACT

Context: Epigallocatechin-3-gallate (EG), the main active flavonoid in green tea, has well-known anti-inflammatory, antioxidant, and anti-apoptotic activities.

Objective: The EG protection against testicular injury induced by cisplatin was studied in Sprague–Dawley rats.

Materials and methods: Cisplatin (10 mg/kg, i.p.) was given as a single injection to rats. EG was given at 40 and 80 mg/kg/day, i.p., for 5 days, starting the same day of cisplatin insult. Serum testosterone, and testicular malondialdehyde, total antioxidant status, nitric oxide, interleukin-6, interleukin-1\textsuperscript{\beta}, cytochrome C, Bax/Bcl-2 ratio, and caspase-3 were measured. In addition, testicular histopathological examination and immunohistochemical expression of testicular tumour necrosis factor-\textalpha were evaluated.

Results: Cisplatin, compared to the control, significantly decreased serum testosterone (6.48 ± 0.7 vs. 50.8 ± 4.91 ng/10 mL), and testicular tissue antioxidant status (17.3 ± 1.21 vs. 64.12 ± 5.4 \mu mol/g), and significantly increased interleukin-6 (85.81 ± 6.11 vs. 38.2 ± 2.79 pg/100 mg), interleukin-1\textsuperscript{\beta} (98.09 ± 8.31 vs. 32.52 ± 2.08 pg/100 mg), malondialdehyde (74.5 ± 5.88 vs. 23.8 ± 1.91 nmol/g), nitric oxide (104.98 ± 8.5 vs. 52.68 ± 5.12 nmol/100 mg), cytochrome C (5.97 ± 0.33 vs. 1.6 ± 0.99 ng/mg protein), Bax/Bcl-2 ratio (4.01 ± 0.38 vs. 0.71 ± 0.0), and caspase-3 (3.2 ± 0.21 vs. 0.98 ± 0.08 O.D. 405 nm) in rat testes. EG (40 and 80 mg/kg, respectively) caused significant increases of serum testosterone (33.9 ± 2.89 and 47.88 ± 4.4 ng/10 mL), and testicular antioxidant status (47.1 ± 3.92 and 58.22 ± 3.58 \mu mol/g), and significant decreases of interleukin-6 (57.39 ± 4.2 and 48.18 ± 3.98 pg/100 mg), interleukin-1\textsuperscript{\beta} (65.12 ± 5.88 and 41.96 ± 3.51 pg/100 mg), malondialdehyde (42.3 ± 3.9 and 28.67 ± 2.49 nmol/g), nitric oxide (70.6 ± 6.79 and 61.31 ± 5.18 nmol/100 mg), cytochrome C (3.4 ± 0.27 and 2.21 ± 0.18 mg protein), Bax/Bcl-2 ratio (1.49 ± 0.14 and 1.1 ± 0.09), and caspase-3 (2.1 ± 0.17 and 1.48 ± 0.13 O.D. 405 nm) in testes of cisplatin-treated rats. Additionally, both doses of EG significantly ameliorated the histopathological injury and reduced tumour necrosis factor-\textalpha expression in rat testes.

Conclusion: EG can afford testicular protection in cisplatin-challenged rats by its antioxidant, antinitrative, anti-inflammatory and antiapoptotic effects.

Introduction

Cisplatin (CN) is a commonly used antineoplastic agent in treatment of solid malignancies, including testicular tumours. Despite high efficacy, its usefulness is limited by multiple organ toxicities, such as kidneys, liver and reproductive system. Testicular toxicity and dysfunction is a well-known adverse effect of CN in cancer treatment (Reddy et al. 2016; Soni et al. 2016). Testicular atrophy, temporary or permanent reduction in number, viability, and motility of sperms, inhibition of spermatogenesis, and infertility, were reported with CN in human patients and animal studies (Kaya et al. 2015; Hamza et al. 2016). The precise mechanisms responsible for CN-induced gonadal toxicity are not well elucidated. Growing evidence indicates that increased reactive oxygen species (ROS), exhaustion of endogenous antioxidants, and membrane lipid peroxidation with increased production of malondialdehyde (MDA) are involved in the pathogenesis of this condition (Soni et al. 2015; Simsek et al. 2016). Additionally, activation of inflammatory responses, increased production of inflammatory mediators, as tumour necrosis factor-\textalpha (TNF-\textalpha), interleukin-1\textbeta (IL-1\textbeta), and interleukin-6 (IL-6), and reactive nitrogen species (RNS), as nitric oxide (NO), aggravate CN-induced testicular tissue injury leading to necrotic and apoptotic cell death (Sherif et al. 2014; Hussein et al. 2015). Previous investigations showed that CN testicular toxicity was significantly ameliorated by anti-inflammatory and antioxidative agents (Zhao et al. 2014; Reddy et al. 2016; Simsek et al. 2016).

Epigallocatechin-3-gallate (EG) is the main active polyphenolic compound isolated from green tea. It has well-known anti-inflammatory, antioxidative, antiapoptotic, and anticancer activities. Previous investigations revealed that EG significantly prevented CN-induced nephrotoxicity in rats and mice by...
inhibiting inflammation, oxidative stress, and apoptosis (Sahin et al. 2010; Chen et al. 2015). In addition, previous studies showed that EG-enhanced testosterone production in rat Leydig cells (Yu et al. 2010), protected testicular seminiferous tubules and spermatogenesis in rats and mice during testicular torsion-detorsion (Al-Maghrebi et al. 2012; Al-Ajmi et al. 2013), preserved spermatogenesis in mice exposed to ionizing radiation-induced testicular injury (Ding et al. 2015), and protected testicular tissue against toxicity induced by di-(2-ethylhexyl) phthalate (Al-Maghrebi et al. 2012; Al-Ajmi et al. 2013). Therefore, the present work was done to investigate the possible protective effect of EG against cisplatin-induced testicular injury in rats.

Materials and methods

Drugs

CN and EG were purchased from Sigma-Aldrich, St Louis, MO. Both agents were dissolved in physiological saline. Selection of CN and EG doses was done based on previous reports (Ge et al. 2015; Hamza et al. 2016).

Animals

Forty-four Sprague–Dawley male rats (250–300 g weight) provided by the Animal House, College of Medicine, King Faisal University. Animal were housed at 25 °C, 45% humidity, 12 h light/dark cycle, and left to adapt for 7 days before conducting the study. Rats were supplemented with commercial chow and tap water ad libitum. The study followed the International guidelines of use and care of laboratory animals.

Study design

The rats were randomly divided into 4 groups (n = 11, each) as follows:
- First: control received a single i.p. injection of physiological saline.
- Second: received a single i.p. injection of CN (10 mg/kg), and an i.p. injection of physiological saline (EG vehicle) daily for 5 days.
- Third and fourth: received a single i.p. injection of CN, and EG (40 and 80 mg/kg/day, respectively, i.p.) for 5 days, starting the same day of CN injection (the first EG dose was given 6 h after CN).

Sample collection and biochemistry

Rats were euthanized on the 6th day following CN injection by thiopental (100 mg/kg, i.p.). Samples of blood were collected via a heart puncture, and serum testosterone was assayed by an ELISA kit (Enzo Life Sciences, Lausen, Switzerland). The testes were dissected and the right testes were homogenized in cold potassium phosphate buffer (pH 7.4, 0.05 M), and homogenates were centrifuged at 4000 rpm for 10 min at 4 °C. Thereafter, colorimetric kits were used for measurement of MDA, NO, and total antioxidant status (TAS), in the testicular homogenate supernatant (BioVision, Milpitas, CA). Additionally, ELISA kits were used to measure IL-6 and IL-1β (R&D Systems, Minneapolis, MN), and Bax and Bcl-2 (LifeSpan BioSciences, Seattle, WA).

Caspase-3 activity was also measured by a colorimetric kit (R&D Systems, Minneapolis, MN). The test principle is to release p-nitroaniline (pNA) via degradation of a specific enzyme substrate. Absorbance of pNA was measured by spectrophotometry at 405 nm, and pNA absorbance of different groups was compared with that of control.

A homogenate portion was centrifuged at 10,000 rpm for 30 min at 4 °C. Cytosolic fraction (the supernatant) was used to measure cytochrome C by an ELISA kit (R&D Systems, Minneapolis, MN).

Histopathology procedure

The left testes were fixed in Bouin’s solution, dehydrated in alcohol, and embedded in paraffin. Five micrometres thick sections were cut, and stained with haematoxylin and eosin (H&E). In a blinded manner, the slides were examined by a pathologist under light microscope.

Testicular injury was assessed by a semi-quantitative analysis of seminiferous epithelium damage, tubular necrosis, interstitial oedema, and haemorrhages using a scale from 0 to 3, where 0 means no abnormal findings, and 3 means severe abnormal findings (Erpek et al. 2007). Spermatogenesis was also assessed using a scale from 1 to 10, where 10 reflects normal spermatogenesis, and 1 reflects atrophy with no spermatogenesis, as previously described (Johnsen 1970).

Immunohistochemistry procedure

The sections were deparaffinized and rehydrated, and H2O2 (3%) in methanol was used to inhibit endogenous peroxidase. Sections were pretreated in citrate buffer (pH 6.0, 10 mM) in a microwave, and incubated with rabbit polyclonal antibody for rat TNF-α (US Biological, Swampscott, MA, 1:100). Sections were incubated with biotinylated goat anti-polyvalent, streptavidin peroxidase, and lastly with DAB as chromogen. Counterstaining by haematoxylin was done, and immunostaining was detected by light microscope using a digital imaging software program (cellSens, Olympus Corporation, Center Valley, PA) to evaluate immunoreactive area (μm²) in five different microscopic fields. The mean ± S.E.M. of each group was calculated.

Statistical procedure

Results are presented as mean ± S.E.M. Analyses were done by one-way ANOVA test followed by Tukey test for post hoc comparisons using GraphPad Prism software program (version 5, San Diego, CA), and significance was at p < 0.05.

Results

Biochemistry findings

Figure 1 shows that a single dose of CN (10 mg/kg, i.p.) significantly decreased serum testosterone, and testicular TAS, and significantly increased testicular MDA, NO, IL-6, and IL-1β in rats in comparison with the control group (p < 0.05). Administration of EG (40 mg/kg/day, i.p., for 5 days) significantly increased serum testosterone, and testicular TAS (p < 0.05) in rats received CN, but still less significant than the control level (p < 0.05). EG
(40 mg/kg) also significantly reduced MDA, NO, IL-6, and IL-1β (p < 0.05) in the testes of rats challenged with CN. However, MDA, IL-6, and IL-1β levels obtained with EG (40 mg/kg) were significantly higher than the control values (p < 0.05) (Figure 1). On the other hand, EG (80 mg/kg/day, i.p., for 5 days) significantly increased serum testosterone, and testicular TAS (p < 0.05), and significantly decreased testicular MDA, NO, IL-6, and IL-1β (p < 0.05) in rats received CN. All the measured parameters observed with EG treatment (80 mg/kg) were comparable with the corresponding control values without significant difference (Figure 1).

Regarding apoptotic indicators, CN administration caused significant elevations of cytosolic cytochrome C, Bax/Bcl-2 ratio, and caspase-3 (p < 0.05) in rat testes (Figure 2). EG (40 mg/kg) significantly decreased testicular cytosolic cytochrome C, Bax/Bcl-2 ratio, and caspase-3 (p < 0.05) in CN-challenged rats. By comparing the values of EG (40 mg/kg) with the control values, it was found that Bax/Bcl-2 ratio was comparable to, however, cytochrome C and caspase-3 were significantly higher (p < 0.05) than the control levels (Figure 2). Treatment with EG (80 mg/kg) significantly decreased the CN-induced elevations of testicular cytosolic cytochrome C, Bax/Bcl-2 ratio, and caspase-3 (p < 0.05), without significant difference from the control levels (Figure 2).

**Histopathology and immunohistochemistry findings**

Figure 3 displays that CN caused marked distortion of seminiferous tubules, vacuolization, necrosis and desquamation of tubular epithelium, reduction or absence of spermatogenesis, and edema of interstitium. EG (40 and 80 mg/kg) resulted in significant protection of rat testes, reduced testicular injury score, and preserved spermatogenesis, compared to CN group non-treated with EG (p < 0.05). The two doses of EG significantly reduced the testicular injury score to the control level, but only EG (80 mg/kg) increased the spermatogenesis score to a comparable level with the control (Figure 3).

Figure 4 displays a significant elevation of TNF-α expression in testes of CN-treated rats compared to control group (p < 0.05). On the contrary, both doses of EG resulted in significant decreases of testicular TNF-α expression in comparison with rats receiving CN (p < 0.05), and the results of the two EG doses were comparable to the control (Figure 4).

No immuopositivity was detected in CN group not treated with EG when the primary antibody was replaced by normal rabbit serum pointing to the antibody specificity (non-included figures).

**Discussion**

The role of oxidative stress and increased production of ROS, as hydroxyl radicals, hydrogen peroxide, and superoxide anion, in the development and progression of reproductive organs injury induced by CN were reported in previous studies. Subsequent exhaustion of cellular antioxidant defenses, as reduced glutathione, and antioxidant enzymes, leads to exaggerated lipid peroxidation of mitochondrial and sperm membranes, and increased production of MDA (Rezvanfar et al. 2013; Saral et al. 2016). Excess ROS generation also up-regulates inflammatory cascades with upstream production of inflammatory mediators, mainly TNF-α and subsequently IL-6 and IL-1β. This magnifies gonadotoxicity and spermatoxicity leading to testicular dysfunction and reduced fertility (Yamaguchi et al. 2008; Sherif et al. 2014). Previous reports clearly demonstrated that EG effectively scavenged ROS, prevented initiation and propagation of oxidative chain reactions, and inhibited the production of inflammatory cytokines in different models of oxidative injury and inflammation, including those caused by CN administration (Zou et al. 2014; Chen et al. 2015). This is in agreement with present work, in which EG preserved testosterone production, spermatogenesis, and testicular TAS, and inhibited the increase of MDA, TNF-α, IL-6 and IL-1β in rat testes exposed to CN toxicity.

Exaggerated nitrative stress was also reported in CN-mediated gonadal injury. Increased activity of inducible nitric oxide synthase, and excess production of RNS, as NO and peroxynitrite, resulted in nitration and damage of macromolecules of the cell (Ilbey et al. 2009; Hamza et al. 2016). In consistence with the present investigation, it was reported that EG suppressed cellular nitrative stress by preventing excess production of RNS. This was attributed to the ability of EG to block inflammatory responses, and enzymatic activity of inducible nitric oxide synthase (Al-Maghrebi et al. 2012; Zhang et al. 2015).
Figure 3. H&E (200×) photomicrographs of rat testes from: (a) control group showing normal testicular structure; (b) cisplatin (CN) group showing necrosis of seminiferous tubular cells, desquamation of tubular epithelium (black arrow), vacuolization (black head), absence of spermatogenesis, oedema of interstitium (white arrow); (c and d) Epigallocatechin-3-gallate 40 mg/kg (EG 40) + CN, and EG 80 mg/kg (EG 80) + CN, respectively, showing marked improvement with minimal damage; (e) spermatogenesis score; (f) testicular injury score. Results are mean ± S.E.M., *p < 0.05 vs. control group, **p < 0.05 vs. CN group.

Figure 4. Immunohistochemistry (200×) of tumour necrosis factor-α (TNF-α) in rat testes from: (a) control group with minimal TNF-α expression; (b) cisplatin (CN) group showing a significant elevation of TNF-α reactivity; (c and d) epigallocatechin-3-gallate 40 mg/kg (EG 40) + CN, and EG 80 mg/kg (EG 80) + CN, respectively, showing significant decreases in TNF-α positivity; (e) immunoreactive area (μm²). Results are mean ± S.E.M., *p < 0.05 vs. control group, **p < 0.05 vs. CN group.
The Bcl-2 family proteins control cell survival and death by regulating the mitochondrial apoptotic pathway. The Bcl-2 protein is an anti-apoptotic factor which maintains mitochondrial membrane integrity. On the other hand, Bax protein, a pro-apoptotic factor is increased in conditions of oxidative stress, and causes disruption of membrane permeability with subsequent release of cytochrome C in the cytosol (Yang et al. 2015; Ding et al. 2016). Cytosolic cytochrome C interacts with the apoptotic protease-activating factor forming apotosome, which finally induces caspase-3, the key factor in execution of cell apoptosis. This leads to DNA degradation, chromatin condensation, and bio-membrane protein destruction (Abdelrazeek et al. 2016).

Reproductive toxicity of CN is mediated via activation of mitochondrial apoptosis, and by increasing Bax/Bcl-2 ratio (Turk et al. 2011; Hussein et al. 2015). In the present investigation EG inhibited apoptosis by reducing cytosolic cytochrome C, ratio of Bax/Bcl-2, and caspase-3 in the testes of CN-treated rats. Similarly, the anti-apoptotic activity of EG proved in previous studies was attributed to its antioxidative and anti-inflammatory effects (Al-Ajmi et al. 2013; Ding et al. 2015).

Regarding histopathological findings, CN caused disruption of seminiferous tubules, necrosis, vacillation, and desquamation of tubular epithelium, oedema of interstitium, and reduced spermatogenesis. This is in accordance with previous reports, which showed similar histological changes in testicular tissue following CN administration (Hamza et al. 2016; Reddy et al. 2016). EG significantly preserved the integrity of rat testes, and maintained spermatogenesis, in a dose-dependent manner, after CN toxicity.

Conclusions

EG provided significant protection against testicular toxicity and spermototoxicity in rats exposed to CN. This is attributed to the beneficial properties of EG, such as inhibition of oxidant/nitrative stress, inflammation, and apoptosis.

Disclosure statement

There are no conflicts of interest.

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