Protective effects of subchronic caffeine administration on cisplatin induced urogenital toxicity in male mice

M Khazaei1, P D Bayat2, A Ghanbari1*, S Khazaei1, M Feizian3, A Khodaei3 & H A S Alian3

1Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, P.O. Box 1568, Kermanshah, Iran
2Department of Anatomy, Arak University of Medical Sciences, Arak., Iran
3Student Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran

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In Cisplatin treated group, the degeneration intensity of the kidneys the diameter of seminiferous tubules as well as the apoptotic index in testes and kidney were increased. In Caffeine+Cisplatin treated groups, the total body weight, the weight of testes and kidneys and also the histopathological data did not show significant differences. The motility of sperm in cisplatin group reduced but in Caffeine+Cisplatin groups this parameter was increased. These data suggest that caffeine recovers toxicity induced by cisplatin in both kidneys and testes of mice.

Keywords: Apoptosis, Caffeine, Cisplatin, Mice, TUNEL

Cis-diaminedichloroplatinum (II) or cisplatin (CIS) is a potent antineoplastic drug for the treatment of many solid tumors. The clinical usage of the drug is limited, because only high doses are effective and that lead to adverse side-effects. Also, low doses of the drug induce pathological signs in animals1,2.

Nephrotoxicity is one of the most potent side-effects of cisplatin and occurs in over 30% of treated patients3. The nephrotoxicity of cisplatin is shown by tubular cell death, tissue damage, and the loss of renal function or acute renal failure in rat4,6. Male reproductive system is another target of cisplatin induced toxicity. Cisplatin induces azoospermia in human7 and sperm morphology and motility alterations in mouse8,9. Testicular toxicity of cisplatin is explained by changes in structure and functions of Sertoli and Leydig cells10,11, germ cell apoptosis12 and increase in microtubule stability13. At molecular level, the formation of oxidative agents such as reactive oxygen species (ROS)14, inducible nitric oxide synthase (iNOS)15 and also decrease in antioxidant enzymes are considered as the main mechanisms of nephrotoxicity of cisplatin16.

Caffeine as a major constituent of coffee, tea and other beverages is a widely used dietary compound17. The effects of caffeine on healthiness have attracted the researcher’s attention to know probable hazards or benefits of this compound. Epidemiological studies showed the associations between intake of coffee and unhealthy behaviors, such as cigarette smoking and physical inactivity and also reductions in the risk of several chronic diseases18,19.

Caffeine has significant abilities to scavenge highly reactive free radicals and excited states of oxygen and to protect crucial biological molecules against these species20. On the other hand, caffeine and its catabolic products, theobromine and xanthine, show prooxidant activity19. These are the possible reasons why caffeine acts as a radioprotector against oxygen-dependent ('oxic') pathway of radiation damage and as an antimutagen/anticarcinogen under certain conditions20,21.

The effect of caffeine on biological systems has been examined and the results differ according to the dose, tissue and duration of treatment. Further, acute treatment and in vitro administration of caffeine increases the motility of epididymal sperm in the rat22. Chronic dietary caffeine (0.1%) has detrimental effects on spermatogenesis and ejaculatory response of roosters23 and scattering vacuolar degeneration of spermatogenic cells in rat testes (0.5%)24. Further, chronic in take of caffeine (30 or 60 mg/kg/day) for four reduces sizes of the seminiferous tubules consecutive weeks and inhibits the spermatogenesis of rabbits25. Surprisingly, although chronic intake of caffeine (30 mg/kg) by male rats, affects sperm parameters of the next generation26, this dose not induce nephrotoxicity in rat after 90 days of administration27.

*Correspondent author
Tel/Fax: +98-831-4281563
E-mail: aghanbari@kums.ac.ir
In kidney, either chronic (3 mg of caffeine/kg body mass/24 h) or acute (0.2%) oral administration of caffeine has diuretic effects on the examined rats by increasing diuresis, resulting in dehydration and also elevating nitric oxide $^{28}$.

The present study has been conducted to find the protective effects of subchronic caffeine administration on cisplatin induced toxicity in mice. In this regard, semi quantitative analysis of the diameter of seminiferous tubules, degeneration intensity of kidneys and estimating of apoptotic cells of kidneys and testes have been performed.

**Materials and Methods**

**Animals**—Inbred male balb/c mice (27 ± 2 g; 36) were obtained from a closed bred colony at the University. All animals received care as recommended by the Research Committee of the University. The mice were maintained on a regular diet and water at a 12:12h light: dark cycle without any stressful stimuli at 23 ± 2 °C. The animals were provided with standard diet pellets and water ad libitum. Experiments were started after one week of adaptation. All experiments were carried out according to the Guidelines of the Animal Care and Use Committee at the University.

**Drugs**—Cisplatin (EBEWE Pharma, Unterach, Austria) was dissolved in saline in darkness, 10-15 min before use and an interaperitoneal injection (5.5 mg/kg) was given at the forth day of experiment $^{29}$. Caffeine (Sigma, Co, St Louis, MO, USA, C0750) was dissolved in drinking water (0.1, 0.5, and 1%) and orally administered from day one for 15 days. To determine the amount of water intake, each animal was housed individually for a week prior to start the experiments $^{30,31}$.

**Experimental procedure**

The mice were divided into 6 groups (of 6 each). The first group (control) received normal saline (0.9% NaCl). Experimental groups were treated with cisplatin (E1), cisplatin+ 0.1% of caffeine (E2), cisplatin+ 0.5% of caffeine (E3), cisplatin+ 1% of caffeine (E4) and finally 1% of caffeine without cisplatin (E5). Body weights of the mice were recorded initially and at the end of the experimental procedure (day 15). Weights of the kidneys and testes were also noted.

**Sperm parameters assessment**

The left caudae epididymis were dissected and were kept for 30 min at 37 °C to promote the release of sperm into the Petri dish containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12). Then, sperm were collected with a micropipette (aliquot 20 µl) on a slide for motility analysis. Sperm motility was determined by counting all progressive sperm, the non-progressive and the immotile sperm in the same field. In each preparation, at least 200 sperm was counted. This motility assessment was repeated in a new preparation from the same semen sample $^{32}$.

**Histopathological observations**

Kidneys and testes were fixed in 10% neutral buffered formalin. The paraffin blocks were cut in 5 µm thick sections which were stained with hematoxylin–eosin (H&E) and examined by light microscope at x40. The diameters of the seminiferous tubules were measured using a specialized software package (AE-3; Motic S.L.U., Barcelona, Catalonia, Spain). For this reason, 50 random tubular cross-sections per testicular section were examined at x40, totaling 200 seminiferous tubule sections per animal $^{33}$.

A pathologist carried out a semiquantitative analysis of the kidney sections in a blind manner. Histopathological analyses of the kidneys were based on the dilatation of Bowman’s capsule, medullar congestion, and dilatation of collecting tubules for kidney $^{34}$.

This scale used was $\text{A} = \text{weak in} \leq 25\% \text{ of tissue}; \text{B} = \text{mild in} \geq 25 – \leq 50\% \text{ of tissue}; \text{C} = \text{moderate in} \geq 75\% \text{ of tissue}; \text{and D} = \text{very strong in} \geq 75– \leq 100\% \text{ of tissue}$. The average degeneration intensity was calculated as $[(\text{A} \times 1) + (\text{B} \times 2) + (\text{C} \times 3) + (\text{D} \times 4)]/(\text{A} + \text{B} + \text{C} + \text{D})$ and reported as follows: $+ = 0.00–1.00; ++ = 1.01–2.00; +++ = 2.01–3.00; \text{and++++}=3.01–4.00$.

The scores were derived from semi-quantitative score using light microscopy on the preparations from each animal, and were reported as follows: none = –, mild = +, moderate = ++, severe = +++ and very strong = ++++.

**TUNEL assay**

Apoptosis was assessed by terminal deoxynucleotidyl transferase (TdT)- mediated deoxy-UTP nick end labeling (TUNEL) assay by using In Situ Cell Death Detection Kit, POD (Roche Diagnostics Deutschland GmbH, Mannheim, Baden-Württemberg, Germany; 11684809910).
The paraffin sections were dewaxed and rehydrated by standard methods. Proteases were added and incubated with 5% of appropriate normal serum for 30 min at 37 °C. The slides were washed in phosphate buffered saline (PBS). The sections were permeabilised (2 min, on ice) and incubated with TUNEL reaction mixture (60 min, 37 °C). Anti-fluorescein-AP was added and incubated (30 min, 37 °C). Subsequently, the sections were washed in PBS and incubated for 20 min with substrate. They were analyzed by light microscopy. Apoptotic index (AI) was calculated by dividing the number of TUNEL-positive cells to total number of the cells in randomly focused fields, and the result was multiplied by 100\(^{35}\).

**Statistical analysis**—All data were analyzed using SPSS 16.0 for Windows XP (SPSS Inc., Chicago, IL). Values were presented as means ± SD. Differences between groups were examined for statistical significance using One-Way analysis of variance (ANOVA) and post hoc Tukey test. Nonparametric Mann-Whitney tests were performed when data were not distributed normally. \(P\) values of 0.05 or below were considered as significant.

**Results**

No death was observed among all groups during the period of the study. The total body weight \((P=0.008)\) (Fig. 1a), and the weight of the kidneys \((P=0.03)\) (Fig. 1b) and testes \((P=0.04)\) (Fig. 1c) in cisplatin treated group (E1) were reduced.

**Histopathological data**

Testicular sections showed that the diameter of the seminiferous tubules were reduced in cisplatin treated mice \((P=0.006)\). No significant alteration in the diameter of seminiferous tubules was seen in caffeine involved groups (Fig. 1d). The kidney sections showed a high degree of nephrotoxicity of cisplatin \((P=0.001)\). There were no significant changes in other experimental groups (Fig. 1e).

Movement analysis of sperms indicated that the total motility increased in E3 \((P=0.04)\), E4 \((P=0.03)\), E5 \((P=0.03)\) while it was decreased in E1 \((P=0.34)\) (Fig. 1f). This analysis also confirmed that progressive sperm movement was increased in E3 \((P=0.02)\), E4 \((P=0.03)\), and E5 \((P=0.03)\) groups (Fig. 1g).

The TUNEL staining sections of the kidneys revealed that apoptosis occurred in both cortex and medulla of cisplatin involved groups (Fig. 2) and evaluated apoptotic index of these sections presented significant increase in E1 group \((P=0.009)\) (Fig. 1h). TUNEL staining sections of the testis showed that both germ cells and Sertoli cells can be induced to apoptosis (Fig. 3) and evaluated apoptotic index of these sections presented significant increase in E1 group \((P=0.008)\) (Fig. 1i).
Discussion

The results showed a significant declination in the weight of animals treated with cisplatin. This data support other studies that indicated reductions in body weight could be attributable to toxic side effect of chemotherapeutic drugs and it suggest that caffeine reliefs the adverse effect of cisplatin.36

The present study showed histological damage in testis and kidney of mice after 14 days of one cytotoxic dose of cisplatin. Studies on animal models have shown that either acute or chronic administration of cisplatin induces adverse effects on kidneys and various male reproductive parameters shortly after exposure.3-15 Moreover, a period of 14 days is needed for repairing the damaged testis of mice admitted to cisplatin.37 Thus, only cytotoxic dose of cisplatin induces histopathological side effects on reproductive system and kidney that irreversibly remains up to 14 days of injection.

There are many ways to protect toxicity of chemotherapeutic drugs. Hormone therapy is a method to enhance recovery of spermatogenesis in chemotherapy treated animals. For this propose GnRH analogue Zoladex,38, testosterone,39 testosterone and oestradiol,40 GnRH and testosterone,41, and GnRH and the anti-androgen flutamide,42,43 were applied before and during the period of treatment of animal with chemo-radiotherapy.

In kidney, experimental prevention of acute cisplatin nephrotoxicity may be obtained with...
intensive hyperhydration\textsuperscript{44}, mainly containing chloride salts\textsuperscript{45} and diuretics\textsuperscript{46}. A large number of studies also reported the beneficial effects of a variety of antioxidants in cisplatin induced nephrotoxicity\textsuperscript{77,48}. Agents such as SOD, dimethyl thiourea and reduced glutathione (GSH) have been shown to reduce the degree of renal failure and tubular cell damage when administered simultaneously with cisplatin\textsuperscript{79}. 

Beside these therapies, herbal extracts have attracted the attention of many researchers for reducing the side effects of chemotherapeutic agents. This attenuation is based on the fact that free radicals that mediate reactions are responsible for a wide range of cisplatin induced side effects. Consequently, anti-oxidants have been shown to protect non-malignant cells and organs against damage by cisplatin\textsuperscript{50-54,5,6}.

Among herbal extracts, ginger rhizome and Roselle are two components that reduce the toxicity of cisplatin on the testis of rats by reducing apoptotic cell death in germ cells and Sertoli cells\textsuperscript{50}.

In the present study, apoptotic DNA fragmentation was determined in kidney and testis using the TUNEL technique. A single dose of cisplatin caused apoptosis in testes (germ cells and Sertoli cells), medulla and cortex of the kidneys. The high rate of apoptosis in the present study suggests that apoptosis is an important mechanism of toxicity of cisplatin. 

Chemotherapy leads to single and double DNA strand breaks, most often followed by cell death. Recent studies have shown the important role of apoptosis in the pathogenesis of cisplatin testicular and renal damage\textsuperscript{52-54,5,6}.

Thus, together with other herbal extracts, caffeine reduces cytotoxicity of cisplatin by decreasing the number of apoptotic cells that can be considered as the antioxidant activity or diuretic effect of caffeine.

Cisplatin but not other platinum compound such as carboplatin, oxaliplatin and nedaplatin induces nephrotoxicity mediated by organic cation transporters certainly organic cation transporter 2 (OCT2). This molecule mediated cisplatin uptake in renal proximal tubular and cochlear hair cells. Ciariomboli et al\textsuperscript{55} suggest that chemotherapeutical protocols aimed to maximize the antineoplastic effect of cisplatin while reducing the risk of toxicities by competing OCT2.

It is suggested that as previously showed that caffeine could interfere with organic cation transporter of intestine\textsuperscript{56}, OCT2 of proximal tubules of nephrons is the other target for caffeine.

In conclusion, the study presents caffeine as an agent for reducing irreversible side effects of cisplatin and discusses three involving mechanism of this effect. Antioxidate, diuretic activities and blocking organic cation transporter. The importance of study is that subchronic and also low doses of caffeine are effective. The data can be used for further investigation about estimation of the dose and duration of usage of these two compounds in combination therapy.

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