Research Paper

Analyzing the effects of zinc on histopathological changes in rat testis caused by cisplatin toxicity

Accepted 5th June, 2017

ABSTRACT

This study aims to investigate to what extent the toxic effect caused by Cisplatin on rat testis can be reversed by zinc that has antioxidant properties. The rats were divided into 4 groups: the control group (Group I; n=6), cisplatin (CIS-Platinum) group (Group II; CP Group, n=6), Zinc (zinc sulphate) group (Group III; Zn Group, n=6) and Cisplatin + Zinc group (Group IV; CP+Zn Group, n=6). The control group was administered intraperitoneal (ip.) (2 ml/kg) saline for 30 days, Cisplatin group was administered; ip. saline solution for the first 20 days, cis-platinum (daily 2 mg/kg) for the following 5 days and saline solution for the last 5 days, Zinc (Zn) group was administered zinc sulphate (3 mg/kg) ip. for 30 days and Cisplatin + Zinc group (CP+Zn) was administered; only zinc sulphate (3 mg/kg) ip. for 20 days, cis-platinum (daily 2 mg/kg) for the following 5 days and only zinc sulphate (3 mg/kg) ip. for the last 5 days. At the end of 30 days, the rats were sacrificed and their testes processed for assessment of germ cell apoptosis (TUNEL method) and the quality of spermatogenesis (Johnsen score system). The germ cell apoptosis were significantly increased in CP treated rats compared with control (P<0.05). Spermatogenesis significantly reduced in CP treated rats compared with those of the control group (P<0.05). Treatment with CP and Zinc resulted in an inhibition effect on germ cell apoptosis and a significant increase in the Johnsen score compared with CP treated mice (P<0.05). Therefore, the application of zinc may serve as a beneficial medication to protect germ cells against apoptosis.

Key words: Cisplatin, zinc, testis, apoptosis.

INTRODUCTION

The importance of various cancer types that occurs in different tissues of the body has been on the increase. Many studies have been conducted worldwide on the early diagnosis, aetiology and pathogenesis of cancer and development of new treatment drugs. On the other hand, the number of studies on increasing the effectiveness of antineoplastic agents and decreasing their side effects has also been increasing (Babu et al., 1995; Seikh-Hamad et al., 1997).

Cisplatin (CP) which is a chemotherapeutic agent was successfully used in clinical oncology against diverse types of cancers. It is an efficient platinum-derived alkylating agent that acts in unspecific phases of the cellular cycle against proliferating and resting cells; it is exerted mainly on the S phase when the DNA synthesis favors CP cross-linking with inter and intra DNA strands. CP interacts primarily with cytosine and guanine rich DNA regions and causing cellular damage (Lirdi et al., 2008).

Spermatogenic cells are very sensitive to the harmful effects of chemotherapeutic drugs. These drugs may cause irreversible damage to the stem cells and can lead to permanent infertility. The most susceptible cells are spermatogonia and spermatocytes that are up to preleptotene stage. CP is spermatoxic (Meistrich et al., 1989; Carter et al., 1993) and with CP-based chemotherapy, most patients become azoospermic but
they mostly recover from spermatogenesis within 4 years (Drasga et al., 1983).

It is generally accepted that DNA damage and subsequent induction of apoptosis may be the primary cytotoxic mechanism of cisplatin and other DNA-binding anti-tumor drugs (Fisher, 1994). Because the final step of apoptosis is characterized by morphological changes in the nucleus, the death signals of the execution phase must be transmitted from the cytoplasm to the nucleus. Thus, the recognition and processing of cisplatin-induced DNA damage through classic apoptosis requires the transmission of a nuclear signal generated at the initiation phase to the cytoplasm for processing through the effector and execution phases. At the end of the execution phase, the apoptotic signal must return to the nucleus to produce internucleosomal DNA degradation (Gonzalez et al., 2001).

Zinc (Zn) is an element of superoxide dismutase, which is an antioxidant enzyme and may induce the synthesis of metallothionein that protects the tissues against free radicals. Today, Zn is known to play important roles in metabolic events, protein, carbohydrate, energy, nucleic acid, lipid, tissue and hem synthesis, gene expression, immune system maturation and embryogenesis (Rostan et al., 2002).

The accumulation of Zn in the testis at high levels is comparable to those in the liver and kidney (Bedwal and Bahuguna, 1994). In human epidemiological studies, the inhibition of spermatogenesis and sperm abnormalities were observed in patients with Crohn’s disease and nutritional disorders, both of which induce Zn deficiency (Bedwal and Bahuguna, 1994; Prasad, 2008).

In vivo experiments in rodents also demonstrated that Zn deficiency can cause severe damage to the testes such as atrophy of the testicular tubules and inhibition of spermatid differentiation (Mason, 1982; Merker and Gunther, 1997). Moreover, there are some reports that exposure to Zn can alleviate testis damage by stresses such as heavy metals, fluoride and heat (Boran and Özkın, 2004). These findings suggest that the testes may harbor a Zn-incorporation system and that Zn itself may exert protective effect against testicular injury and plays an essential role in the maintenance of testicular functions. However, there has been no evidence reported to date that shows any direct effect of Zn upon spermatogenesis in vertebrates (Yamaguchi et al., 2009).

Within this scope, the results of this study may be important to the public health, contribute to the literature, and shed a light to similar studies and different comments.

MATERIALS AND METHODS

Experimental animals

This study was conducted by Necmettin Erbakan University, Meram Medical Faculty, Experimental Research Centre on 24 male Spraque-Dawley rats aged between 10 to 12 weeks and weighing 250 to 300 g (Ethical Committee approval No: 2010-081). Rats receiving a standard diet were exposed to a 12:12 h light-dark cycle at room temperature of 22 ± 2°C and 60% humidity. Rats were chosen as the experimental animals given that these animals are used in the literature and are easy to obtain.

Experimental animal groups

The rats were divided into 4 groups, they are: Control group (Group I; n=6), Cisplatin (cis-Platinum, Merck S3950710) group (Group II; CP Group, n=6), Zinc (Zinc Sulphate, Merck 1150236) group (Group III; Zn Group, n=6) and Cisplatin + Zinc group (Group IV; CP+Zn Group, n=6). The control group was administered intraperitoneal (ip.) isotonic saline for 30 days; Cisplatin group was administered isotonic saline solution for the first 20 days, cis-platinum (daily 2 mg/kg) for the following 5 days and isotonic saline solution for the last 5 days. This dose was selected based on the dosage of 80 to 120 mg/m² of body surface used in the treatment of testicular neoplasms (Einhorn and Donahue, 1977). Zinc (Zn) group was administered zinc sulphate (3 mg/kg) for 30 days (Tuncer et al., 2011) and Cisplatin + Zinc group (CP + Zn) was administered only zinc sulphate (3 mg/kg) ip. for 20 days, cis-platinum (daily 2 mg/kg) for the following 5 days and only zinc sulphate (3 mg/kg) ip. for the last 5 days. At the end of 30 days, the rats were euthanized under high dose Ketalar-Rompun anaesthesia.

Histopathological analysis

At the end of the experiment all the animals were weighed, euthanized under high dose Ketalar-Rompun anaesthesia and their abdominal walls shaved. The scrotum was entered through a mid-line incision; testes were removed, fixed in 10% neutral buffered formalin and blocked after routine histological follow-up procedures. 5 μm serial sections were cut from each block and in order to see the histological changes in the testis tissue, the sections were stained with Periodic Acid Schiff (PAS; Biostain Ready Reagents, RRSK15-100).

The sections were examined under an Olympus light microscope and the Johnsen method (Johnsen, 1970; Jorge et al., 2006) was used to assess the quality of seminiferous epithelium. Johnsen’s method applied a score of 1 to 10 for each tubule cross-section according to the following criteria: 10, complete spermatogenesis and perfect tubules; 9, many spermatozoa present and disorganized spermatogenesis; 8, only a few spermatozoa present; 7, no spermatozoa but many spermatids present; 6, only a few spermatids present; 5, no spermatozoa or spermatids but many spermatocytes present; 4, only a few spermatocytes present; 3, only spermatogonia present; 2, no germ cells but only sertoli cells present and 1, no germ cells and no
sertoli cells present. The Johnsen score per tubule was expressed as Mean ± SEM for each group (Figure 2).

**In situ DNA end labeling method (TUNEL)**

The kidney tissues were dissected, fixed in 10% neutral buffered formalin, embedded in paraffin wax and then cut into 5 µm thick sections. The sections were put on slides coated with poly-L-lysine for *in situ* DNA end labeling method. Detection of DNA fragmentation *in situ* was visualized with the use of the ApopTag Plus Peroxidase *in situ* apoptosis detection peroxidase Kit (S7101-KIT, Millipore), as described by the manufacturer.

The deparaffinized kidney tissue sections were incubated with proteinase K (20 µg/ml). They were subjected to 3% H<sub>2</sub>O<sub>2</sub> for endogenous peroxidase inhibition and incubated with 1X equilibration buffer at room temperature for 30 min. The digoxigenin-labeled dNTP tail was incubated for 1 h with terminal deoxynucleotidyl transferase (Tdt) at 37°C. The sections were washed in stop or wash buffer for 10 min at room temperature, incubated with antidigoxigenin- peroxidase antibody at room temperature for 30 min and stained with diaminobenzidine (DAB) used for peroxidase substrate. Staining was evaluated using a light microscope after counterstaining with methyl green.

**Apoptotic index**

Morphometric analysis of the positive cells in tissue stained by TUNEL method was performed under high power magnification (×400, Olympus DP72) in a blinded fashion. On each slide, 10 seminiferous tubules were randomly selected. All the TUNEL positive and intact cells in those fields were totaled and apoptotic index calculated by means of an average count per slide. Apoptotic index (Figure 4) was calculated according to the formula as stated by Barut et al. (2005):

\[ \text{AI} = \frac{\text{AC}}{\text{AC} + \text{IC}} \times 100 \]

Where:

- Apoptotic index = AI;
- Apoptotic cell number = AC and
- Intact cell number = IC.

**Statistical analysis**

For all the animals, Graph Pad InStat (Graph Pad Software Inc, San Diego, CA, USA) program was used in Johnsen criteria and Tunnel immune reactions statistically. As the groups had a normal distribution, one-way ANOVA was used to compare the values and Tukey’s test used to assess the differences between the groups; p<0.05 was regarded as significant.

**RESULTS**

The testis section of the control group showed that the seminiferous tubules had smooth margins, germ cells had normal appearance in regular organization and the germ cells at all stages of spermatogenesis was presented in regular organization starting from spermatogonium (Figure 1a). The light microscope evaluation revealed that degenerative effect was significant in the testis tissues of CP group. It was observed in the said group that the arrangement of germ cells were corrupt. Sertoli cells and spermatogonia were detached from the basal membrane, the distance between the cells increased along the epithelial wall and there was a chromatin condensation and fractionation near the lumen especially in spermatids.

In the tubule wall, none of the stages of the main cells and spermatogenesis could be recognized (Figure 1b). Light microscope evaluation revealed that both the basal lamina and seminiferous tubule structures of Zn group were similar to those of the control group (Figure 1c). In CP + Zn group, both the basal lamina and seminiferous tubule structures were nearly similar to those of the control group. Compared to CP group, intact spermatogenic cells and non-erupted smooth seminiferous tubule structures were observed. Moreover, degeneration was observed to exist in the interstitial space (Figure 1d).

**TUNEL findings**

Cells whose nuclei were stained in the TUNEL staining method were regarded as apoptotic. The spontaneous apoptosis observed in seminiferous tubules of the control group rats was observed mainly in spermatogonia and marginally in spermatocytes (Figure 3a). In CP group, many apoptotic nuclei were observed nearly in all the spermatogenic series in the seminiferous tubule (Figure 3b). Zn group was similar to the control group (Figure 3c) and in CP + Zn group, apoptotic nuclei were observed in the spermatogenic series in seminiferous tubule but not as much as in the CP group (Figure 3d).

**DISCUSSION**

CP is a chemotherapeutic agent and a genotoxic drug which causes abnormalities in the head of spermatozoa (Ateşşahin et al., 2006) and spermatozoa DNA damage (Khynriam and Prasad, 2003). Other studies conducted on the damage of genotoxic drugs in spermatozoa DNA showed that many drugs such as acrylonitrile (Xu et al., 2003), tamoxifen (Xu et al., 2001), cyclophosphamide (Anderson et al., 1995) and styrene (Simula and Priestly 1992) cause genotoxic effect and spermatozoa DNA
**Figure 1a:** Regular seminiferous tubules with normal germinal epithelium in control rats, the connective tissue stroma was well distributed.

**Figure 1b:** CP group section of the testicles; all epithelial intercellular increasing distance along the wall depending on sequence to germ cell defect, indicate the interstitial space degenerated.
Figure 1c: Section of testicular Zn group; regular interstitial space and regular germ cells.

Figure 1d: CP+Zn group section of the testicles; regular seminiferous tubules and degenerative interstitial space
When Johnsen Score * was compared to the controls and # was compared to CP, a statistical
difference was observed (p<0.05). Data are expressed in average ± standard error (Control, n=6; CP,
n=6; Zn, n=6; CP+Zn, n=6).

Studies in mice showed acute damage to spermatogenesis following intraperitoneal or intravenous
injection of CP. Even low doses of cisplatin are toxic for spermatogonia. At higher doses, CP causes broad activity
in which it kills some cells in all stages including...
Figure 3b: Detection of apoptosis by TUNEL staining in the rat testis; CP Group, a lot of apoptotic nuclei in seminiferous tubules.

Figure 3c: Detection of apoptosis by TUNEL Staining in the rat testis; Zn Group, a little apoptotic nuclei in seminiferous tubules.
spermatids and spermatocytes around the seminiferous tubule lumen. In this activity, CP may also induce Sertoli cell toxicity. Stem cells appear to be relatively resistant to CP-induced toxicity (Meistrich et al., 1982). Current studies have shown a reversible acute damage to spermatogenic function by CP administration. These
results comply with clinical observations (Drasga et al., 1983; Lange et al., 1983; Berthelsen, 1984; Meistrich et al., 1989) and animal experiments (Meistrich et al., 1982).

In compliance with the aforementioned studies, CP was found to have significant degenerative effect on testis tissue. On evaluating the spermatogenesis in the seminiferous tubule wall according to Johnsen criteria, the maximum damage was observed to be in CP group.

Previous studies showed that apoptosis had a significant role in the pathogenesis of CP-induced testicular damage. CP was reported to cause apoptosis in sertoli cells and testicular germ cells. CP-induced testicular damage is associated with high regulation of p53 expression. High p53 protein triggers DNA damage and stops the cell cycle temporarily or causes apoptosis. Thus, it was claimed that CP-mediated apoptosis in testicular epithelium is a very important component of p53 (Amr et al., 2008).

In recent studies, it was claimed that CP may induce germ cell apoptosis. These studies verified that the germ cells stained positive in TUNEL method are apoptotic and CP exposure causes germ cell apoptosis. Germ cell apoptosis is induced rapidly and reaches its peak level during days of CP exposure. It was shown that the germ cell apoptosis remains at the control level 12 days after CP exposure and the damage to seminiferous tubule is a long-term one. Spermatocytes are shown to be more sensitive than spermatagonia to the effects of cisplatin induced by apoptosis (Seaman et al., 2003). Bax protein is a pro-apoptotic protein belonging to the Bcl-2 family. The role of this protein is to prevent apoptosis (Oltvai et al., 1993). TUNEL assay detects apoptosis in situ, identifying early DNA strand breaks in cells undergoing apoptosis (Godard et al., 1999). As DNA breaks are more abundant and less random in apoptotic cells, higher amounts of DNA fragments (Christina et al., 2006) and more intense TUNEL labeling were observed in those cells (Hasegawa et al., 1997; Stumpp et al., 2004). Despite the presence of TUNEL-positive germ cells that have weak labeling, only the intensely labeled ones were scored. The TUNEL method is more selective for detection of apoptosis than for necrosis (Godard et al., 1999) and it is a morphological labeling method with regard to DNA fragmentation in late apoptosis (Barroso et al., 2000).

According to Huang et al. (1990), albeit the levels of testicular testosterone in adult rats were transiently reduced after 5 daily, thirty days old rat testes showed high level injections of cisplatin (2 mg/kg), these levels were sufficient to support complete spermatogenesis; thus, regression of seminiferous epithelium induced by cisplatin could not account for hypoandrogenism. Testicular germ cell sensitivity to cisplatin may be related to their inclination to undergo apoptosis (Seaman et al., 2003). According to Zhang et al. (2001) and Seaman et al. (2003), a single cisplatin dose of 5 mg/kg causes peak apoptotic waves of germ cells at 72 and 24 h in adult mice.

Zinc (Zn) is an essential trace element required for the maintenance of germ cells, progression of spermatogenesis and regulation of sperm motility (Yamaguchi et al., 2009). Zn is a cofactor in many enzymes and proteins that are involved in antioxidant defenses, electron transport, DNA repair and p53 protein expression (Song et al., 2009).

Reportedly, Zn deficiency induces oxidative stress (Oteiza et al., 1996; Ho et al., 2003; Song et al., 2009). Supplementation with Zn was shown to give protection against a range of stress-induced testicular damage (Ozturk et al., 2003; Amara et al., 2008). Oxidative stress occurs when overgeneration of reactive oxygen or nitrogen species (ROS and RNS) overwhelms antioxidant defenses or endogenous antioxidant defense is down-regulated. Oxidative stress induced by Zn deficiency is not only due to the increased generation of ROS and or ROS, but also due to the impairment of antioxidant capacity (Oteiza et al., 1996; Yousef et al., 2002).

The immuno-histochemical evaluations carried out showed that the spontaneous apoptosis in the seminiferous tubules of the control group rats were observed mainly in spermatogonia and marginally in spermatocytes. In CP group, many apoptotic nuclei were observed nearly in all the spermatogenic series in the seminiferous tubule. In CP + Zn group, apoptotic nuclei were observed in the spermatogenic series in the seminiferous tubules but not as much as in the CP group.

As a result, it was found in the present study that administration of 10 mg/kg cisplatin for five days was quite toxic for the testicular tissue; it induced apoptosis and deteriorated the histologic structure.

Conclusion

The administration of zinc which is an essential material and having antioxidant effect for one month was found to undo the damages caused by cisplatin. Therefore, the application of zinc may serve as a beneficial medication to protect germ cells against apoptosis.

ACKNOWLEDGMENT

The study was supported by the Selcuk University Scientific Research Projects Coordination Unit (Project no: 10102041).

REFERENCES


