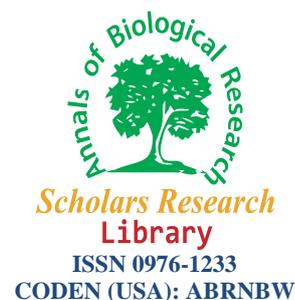




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Annals of Biological Research, 2012, 3 (5):2297-2303
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Toxicity effect of cisplatin –treatment on rat testis tissue

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ABSTRACT

Cisplatin is widely used as an antitumor drug. To reduce its toxic side effects in patients .Cisplatin is a chemotherapy drug. It is used to treat various types of cancers, including sarcomas, some carcinomas , lymphomas, and germ cell tumors. It was the first member of a class of platinum-containing anti-cancer drugs, which now also includes carboplatin and oxaliplatin. These platinum complexes react in vivo, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis. Since cisplatin provokes fertility and induces germ cell apoptosis and necrosis Therefore, we have been tried in this study to evaluate the toxic effects of this drug on Rat testis tissue. In this study we choose 40 adult male Rat. The Rats were divided randomly into 2 groups as Control and Experimental. Rats of the experimental group were injected by Cisplatin as 3 dose (20mg/m²/5days- IP) .The Control group injected by normal Salina. At the termination of experiments the testis were fixed and stained with H&E and Trichrome Masson. Data were analyzed by T-Test and SPSS software.Microscopic study shows changes includes: The diameter of seminiferous tubules and epithelial thickness and the average percentage of tubules with spermatozoa was significant diminished. The spermatogenic cells were significant decreased with degenerative changes. Interstitial testicular edema probably occurred in cisplatin-treated rats .In comparing the average of the parameters in the experimental group with control group, there was significant difference (P<0.001). The study showed that cisplatin-induced germ cell apoptosis in the Rat testes. This study suggests that Cisplatin impairs spermatogenesis and testis function. It is suggested that cisplatin-induced germ cell apoptosis may result in decreased spermatogenesis. These finding will have an important bearing for young receiving.

Key words: Infertility, Cisplatin, Rat, Testis.

INTRUDUCTION

Cisplatin, a chemotherapeutic agent, has been successfully used in clinical oncology against diverse types of cancers (36). It is an efficient platinum-derived alkylating agent that acts in unspecific phases of the cellular cycle (21) against proliferating and resting cells (8); its effect is exerted mainly on the ‘‘S’’ phase, when the DNA synthesis favors cisplatin cross-linking (20) with inter- and intra-DNA strands (14,17,26). Cisplatin interacts primarily with cytosine- and guanine-rich DNA regions (29) and causes cellular damage. Despite its potent antineoplastic action, cisplatin’s major side effects include nephrotoxicity (2), peripheral neuropathy (33), azoospermia (11). In mammals, spermatogonia are sensitive to cisplatin and other different chemotherapeutic drugs (23). Moreover, cisplatin also seems to inhibit Leydig cell testosterone secretion (35) and usual changes in hormonal levels observed during cisplatin treatment may be related to Leydig cell damage (3). Apoptosis normally ensures an optimal number of germ cells that can be supported by Sertoli cells (5) and it is triggered in specific areas of the seminiferous epithelium, controlling spermatogonial population (18). An increase in the frequency of germ cell apoptosis seems to occur in different abnormal circumstances, such as hormone deprivation (5,16,32), experimental cryptorchidism (28), mild hypothermia (5), and exposure to chemotherapeutic drugs (30) and other chemical agents (5,27). The cytotoxic effects of chemotherapeutic drugs on germ cells led researchers to additional methods that could preserve fertility in men undergoing traditional anticancer therapies.The mechanisms responsible for the rapid increases in

levels of gonadotropin and steroid hormones upon administration of cisplatin to rats are not clearly understood. We have been tried in this study to evaluate the toxic effects of this drug on Rat testis tissue.

MATERIALS AND METHODS

Albino rats (150–200 g) of the Wistar strain were obtained from the Animal House, Islamic Azad University. They were maintained on a standard pellet diet and tap water *ad libitum* and were kept in polycarbonate cages with woodchip bedding under a 12 : 12 h light : dark cycle and room temperature 22–24°C. Rats were acclimatized to the environment for 2 weeks prior to experimental use. This study was conducted following the guidelines of the Animal Ethics Committee, Islamic Azad University. Cisplatin solution was freshly prepared, protected from light in a saline solution, and was given in a volume of 1 ml/100 g body weight. Control animals received an equivalent volume of saline based on body weight. Rats were randomly divided into 2 groups ($n = 20$) and were subjected to the following treatment: The control group was treated daily with distilled water; In the Cisplatin-treated group, animals were given a single intraperitoneal dose of Cisplatin (10 mg/kg body weight), used previously to induce testicular toxicity in various animal species (9, 41); Following diethyl ether anesthesia, blood was collected from the retro-orbital plexus. After the animals were killed the testes were removed. For histopathological examination, one testis was immediately fixed in 10% buffered formalin.

Histology

For the histological examinations, small pieces of testis were fixed in 10% neutral phosphate-buffered formalin and the hydrated 5 μm -thick sections were stained with hematoxylin and eosin and trichome masson. Sections were examined under a Nikon light microscope.

Immunohistochemistry

Apoptosis was assessed in deparaffinized sections using the terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling (TUNEL) technique. In this technique, the manufacturer's protocol for the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) was followed. This method detects the DNA fragmentation associated with apoptosis by labeling 3'-OH DNA termini with digoxigenin nucleotides, a process facilitated by terminal deoxynucleotidyl transferase. The labeled fragments are then allowed to bind to anti-digoxigenin antibody conjugated with peroxidase. Color was developed by adding sufficient peroxidase substrate to specimens. Briefly, after deparaffinization and rehydration; tissue sections were treated with 3% hydrogen peroxide for 20 min to diminish non-specific staining. Sections were immersed in 10 mmol/L citrate buffer solution (pH 6.0) in a microwave oven twice for 5 min then incubated with normal goat serum for 20 min. Sections were exposed to the avidin-biotin peroxidase complex (1/400; Dako, Glostrup, Denmark) for 1 h at room temperature. The chromogenic substrate of peroxidase was developed using a 0.05% solution of 3,3'-diaminobenzidine tetrahydrochloride, 0.03% hydrogen peroxide, and imidazole in Tris-HCl buffer (pH 7.6). Sections were counterstained with hematoxylin. The number of apoptotic cells in each section was calculated by counting the number of positive cells in 10 fields per slide at $\times 400$ magnification. This was repeated for all five animals in each group and the average was plotted.

Statistical analysis

Data are expressed as group mean \pm SE. The statistical analysis was carried out using T test, with SPSS software. Analysis of a difference between the means of the treated and control groups was carried out using Dennett's *t* test.

RESULTS

Histological effects

A significant reduction in testicular weight (as a function of total body weight) and total body weight was observed. Control rats showed normal testicular architecture with an orderly arrangement of germinal and Sertoli cells. Cisplatin treatment induced moderate to severe testicular atrophy with degeneration of germ cells in seminiferous tubules and testicular capsule was thickened. The tubules were shrunken and greatly depleted of germ cells. There were depleted numbers of Leydig cells between the tubules. Sertoli cells with few germ cells were observed in the lumen. A drastic reduction in tubular diameter was evident, which correlated with the drop in testicular weight. Testicular cross sections consisted of mostly small "Sertoli cell.

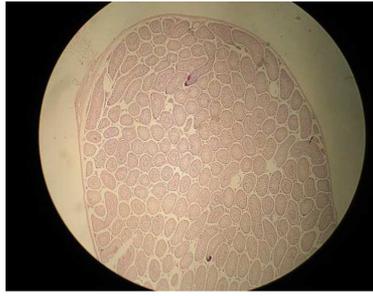


Fig1: Photomicrograph of testicular tissue sections of adult rat from control group. Observe the normal aspect of the seminiferous epithelium, interstitial space and capsule (H&E - x4)

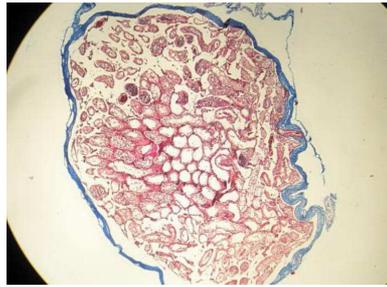


Fig2: Photomicrograph of testicular tissue sections of adult rat from CIS-treated group. Showing tubular atrophy and thickness at testicular capsule (TCM x4)

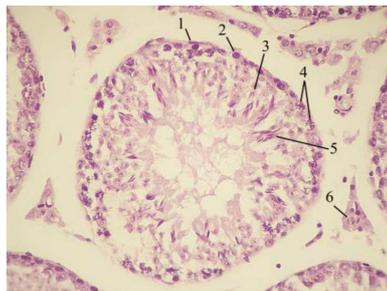


Fig3. Photomicrograph of Seminiferous tubules sections of adult rat from control group. Showing the normal arrangement of germinal cells Sertoli cells and Leydig cells (H&E x40)

1. Myoid cell. 2. Spermatogonia 3. Sertoli cell 4. Primary spermatocyte 5. Spermatozoa 6. Leydig cell



Fig4. Photomicrograph of Seminiferous tubules sections of adult rat from CIS-treated group. Showing the atrophic tubules contained degenerated Sertoli cells with few germ cells (H&E x40)

1. Myoid cell 2. Spermatogonia 3. Sertoli cell 4. Leydig



Fig5: Photomicrograph of testicular tissue sections of adult rat from control group. Observe the normal aspect of the seminiferous epithelium, interstitial space and capsule (TCM – x10)
 1. Testicular capsule 2. seminiferous, tubules 3. Intrstitial space.

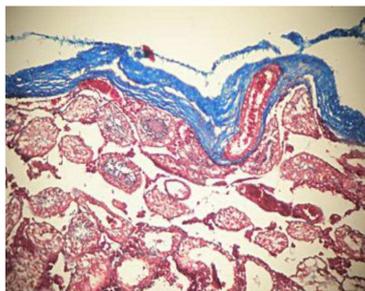


Fig 6. Photomicrograph of testicular tissue sections of adult rat from CIS-treated group. Showing tubular atrophy and thickness at testicular capsule (TCM-x10).
 1. testicular capsule 2. seminiferous tubes. 3. Intrestital space.

Results of quaititive parameters showed us that there was a significant decrease at the seminiferous tubules epithelium height at treatment group ($P < 0.001$) & significant increase at intermediate space distance & testicular capsule thickness ($P < 0.001$) (table 1).

Table 1: Mean of total parameters of testicular tissue (μm), after injection (IP) by cisplatin. Any each parameter was written by (mean \pm SEM), (n=20)

	Seminiferous tubules diameter thickness	Seminiferous tubules epithelium height	Intermdiate space distance	Testicular capsule thickness
Number	20	20	20	20
Control group	0/06 \pm 4/28	0/56 \pm 18/5	0/53 \pm 8/75	0/35 \pm 8/8
Exprimental group	0/1 \pm 2/96 *	0/48 \pm 8/3 *	0/57 \pm 14/8 *	1/65 \pm 24/4 *

* shows the significant difference with control group.($P < 0.001$)

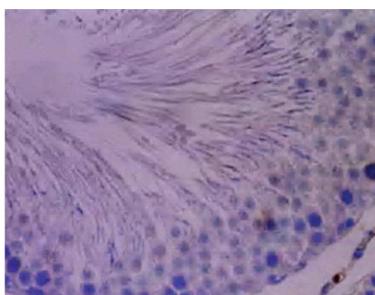


Fig 7. Terminal deoxynucleotidyl transferase-mediated triphoshate nick-end labeling (TUNEL)-positive cells in seminiferous tubules of rats treated with vehicle from controlgroup(x400).

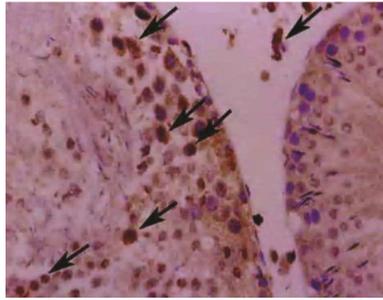


Fig8. Terminal deoxynucleotidyl transferase-mediated triphosphate nick-end labeling (TUNEL)-positive cells in seminiferous tubules of rats treated with vehicle from CIS-treated group. (x400). Brown staining indicates TUNEL-positive cells. TUNEL-positive cells are denoted by arrows.

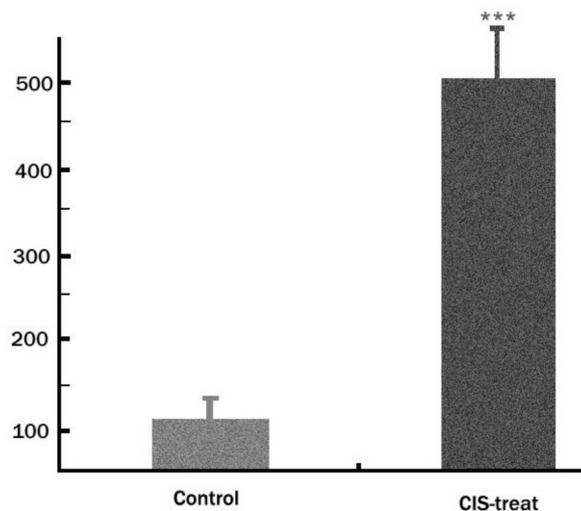


Fig9. The semiquantitative analysis, show variable levels of apoptosis in experimental group. The number of apoptotic cells in each section was calculated by counting the number of TUNEL-positive cells in 10 fields per slide at $\times 400$ magnification. This was repeated for all 20 animals in each group and the average was plotted. Brown staining indicates TUNEL-positive cells. TUNEL-positive cells are denoted by arrows (counterstained with hematoxylin, Bar = 10 μm). *** $P < 0.001$, compared with CIS-treated group.

Apoptotic cell death

TUNEL assay was used to identify apoptotic cells of seminiferous tubules. Brown staining, indicating TUNEL-positive nuclei, was visible in seminiferous tubules of control and Cisplatin-treated animals. However, TUNEL-positive cells were significantly ($P < 0.001$) increased in the Cisplatin-treated group compared to the control group. The number of apoptotic cells in CIS treatment group was calculated by counting the number of TUNEL-positive cells in 10 fields per slide at $\times 400$ magnification.

DISCUSSION

In this study has shown the important role of apoptosis in the pathogenesis of CIS testicular damage and apoptosis of germ cells has been recently reported as a mechanism responsible for the toxic damage to spermatogenesis. The cytotoxic effects of cisplatin and other chemotherapeutic drugs result, in part, from their interactions with DNA (21, 39, 40). DNA-platinum covalent adducts inhibit fundamental cellular processes including replication, transcription, translation, and DNA repair (39). CIS was reported to cause apoptosis to testicular germ cells and Sertoli cells (9,41). In this study, apoptotic DNA fragmentation was determined in testicular tissue using the TUNEL technique and in epididymal sperm using cytometric assessment of DNA damage. A single dose of CIS caused apoptosis in testes (germ cells and Sertoli cells) and in epididymal sperms. Consistent with the results of apoptosis, histological changes were observed in the CIS-treated animal group. The high proportion of apoptosis in the present study suggests that apoptosis is an important mechanism that might account for the marked loss of spermatogenic cells in the CIS-intoxicated testes. The CIS-induced testicular damage was also associated with upregulation of p53 expression. Elevation of p53 protein expression in response to DNA damage triggers either a transient cell cycle

arrest or apoptosis (13,31) Sperms respond to exposure to a DNA-damaging agent by elevating p53 protein levels (38). It is therefore suggested here that p53 is a necessary component in the CIS-mediated apoptotic pathway of testicular epithelia. Apoptosis is a naturally occurring form of programmed cell death, which has the biological function of controlling cell number (19) under physiological conditions. Spontaneous cell death maintains homeostasis in the testis, and it is responsible for the high loss of germ cell in rat seminiferous epithelium (5, 6,21). It is characterized by biochemical features (34) and DNA cleavage leading to typical morphology (1). Some external stimuli such as DNA damaging agents, hormonal disturbance, ionizing radiation, ischemia, and chemical toxic agents can induce germ cell apoptosis (15, 25, 27, and 30). TUNEL assay detects apoptosis in situ identifying early DNA strand breaks in cells undergoing apoptosis (12). As DNA breaks are more abundant and less random in apoptotic cells, higher amounts of DNA fragments (10) and more intense TUNEL labeling are observed in those cells (15,30). Despite the presence of TUNEL-positive germ cells with weak labeling, only those intensely labeled were scored. The TUNEL method is more selective for detection of apoptosis than for necrosis (12), and it is a morphological labeling method with regard to DNA fragmentation in late apoptosis (4).

Exposure to cisplatin results in impaired spermatogenesis, azoospermia, and, sometimes, permanent infertility in male patient's. Recent studies has shown the important role of apoptosis in the pathogenesis of CIS testicular damage (7). In addition to its role in normal testicular physiology (7), apoptosis of germ cells has been recently reported as a mechanism responsible for the toxic damage to spermatogenesis. CIS was reported to cause apoptosis to testicular germ cells and Sertoli cells (9,41). In this study, apoptotic DNA fragmentation was determined in testicular tissue using the TUNEL technique and in epididymal sperm using cytometric assessment of DNA damage. A single dose of CIS caused apoptosis in testes (Germ cells and Sertoli cells) and in epididymal sperms. Consistent with the results of apoptosis, histological changes were observed in the CIS-treated animal group. The high proportion of apoptosis in the present study suggests that apoptosis is an important mechanism that might account for the marked loss of spermatogenic cells in the CIS-intoxicated testes. The current study shows that histological damage in testis is associated with increase in testicular LP. Several studies have shown that CIS toxicity in kidney is mediated by depletions of anti-oxidants and elevations of LP (24,37). CIS has also been suggested to generate free radicals by interaction with DNA (22). Therefore, overproduction of free radicals and hence oxidative stress might account, at least in part, for testicular injury associated with CIS treatment. Recently, much attention has been focused on the protective effects of anti-oxidants and naturally occurring substances against oxidative stress damage. In conclusion, this study showed that apoptotic cell death might play an important role in the development of CIS-induced testicular damage.

We present data that implicate an injury to Sertoli cells as a possible mechanism to explain an elevated rate of germ cell apoptosis and consequent infertility.

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