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Disulfiram partially improves oxidative but not androgen status in rats exposed to cadmium

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Abstract: We investigated the effect of disulfiram (DSF) on reproductive toxicity induced by subchronic exposure to cadmium (Cd). We examined the redox status and systemic testosterone changes in the testes and plasma of Cd-treated male Wistar rats. Rats were treated with 1 mg $CdCl_2$/kg body weight (bw)/day (intraperitoneal administration) for 42 days; in the second experimental group, rats were given 178.5 mg DSF/kg bw/day by oral gavage for 21 days; in the third group, after administration of Cd for 21 days, DSF treatment was introduced on day 22 and lasted until day 42, with continuous Cd intake. Each experimental group had a matching control: untreated rats, rats that received for 21 days olive oil, the solvent for DSF; rats that started with olive oil intake from days 22-42. Exposure of rats to DSF modulated the oxidative status in the testes; thus, coexposure increased the Cd-induced reduction in total superoxide dismutase (tSOD), catalase (CAT), glutathione reductase (GR) and total glutathione-S-transferase (tGST) activities, and lowered the Cd-increased superoxide anion radical ($O_2^{\bullet -}$) and malondialdehyde (MDA) concentrations. DSF did not affect testosterone production diminished by Cd, as Leydig cells, once impaired by Cd, could not be reactivated by DSF.

Keywords: cadmium; disulfiram; oxidative stress; testes; rat

INTRODUCTION

Cadmium (Cd) is a widespread environmental and occupational contaminant, with a verified carcinogenic potential [1]. The major causes of occupational exposure are manufacturing and disposing of Cd-containing composites, while the implementation of certain agriculture measures results in food and water contamination with Cd. Smokers are additionally exposed to Cd through tobacco smoke [2]. A very long half-life of Cd (>20 years) in the human body indicates its resistance to mechanisms of detoxification [3].

Cd disrupts endocrine function, including the reproductive system, diminishing semen plasma and semen quality in humans [4,5]. Recently, we reported that subacute exposure to Cd disturbs redox and bioelement homeostasis in the testes of treated rats, and we showed that Cd caused severe morphological changes in testicular tissue and disturbance in steroidogenesis [6]. Cd can cause oxidative stress (OS) by inhibiting antioxidant metalloenzymes responsible for the sequestration of free radicals (FR), including reactive oxygen species (ROS) and reactive nitrogen species (RNS). Free radical overload interferes with normal cell/tissue physiology by inducing lipid peroxidation (LPO), deoxyribonucleic acid (DNA) and membrane protein oxidative/nitrosative damage, which was confirmed in Cd-associated testicular pathophysiology and toxicology [7, 8].

Due to its reducing and chelating properties, the thiol compound diethyldithiocarbamate (DDTC), the metabolite of disulfiram (tetraethylthiuram disulfide, bis [diethylthiocarbamyl] disulfide) (DSF), which has

been used in aversive therapy of alcoholism [9], is capable of modulating cellular antioxidant status [10]. Thus, we hypothesized that DSF administration could terminate the harmful effect of subchronic exposure to Cd on reproductive toxicity in male rats. We studied the influence of DSF on the testicular status – oxidative, morphological and testosterone changes – in rats after exposure to Cd.

MATERIALS AND METHODS

Animals

The experimental animals were treated according to the Guidelines for Animal Study, No.12032014/9 (Ministry of Agriculture and Environmental Protection, Veterinary Directorate, Serbia). Adult male Wistar rats (weights of 220-250g) were kept in cages under standardized housing conditions (ambient temperature: 23±2°C, relative humidity: 55±3% and a light/dark cycle: 13/11 h, respectively), with free access to standard laboratory pellet-food and tap water. The adaptation period of the rats to the laboratory conditions was two weeks and all subsequent procedures were performed between 9:00 and 13:00 h.

Reagents

All reagents and chemicals used in this study were of analytical grade or higher purity and were obtained from Sigma-Aldrich (St. Louis, USA), Merck (Germany), Werfft-Chemie GMBH (Wien, Austria), Hospital Pharmacy of Military Medical Academy (Belgrade, Serbia), Roche (Germany) and Randox Laboratories (UK).

Experimental design

Adult male Wistar rats were divided into four groups (n=6) as follows: (i) control group (C_{1-42}), untreated rats; (ii) group OL_{1-21} included rats that received olive oil, the solvent for DSF for 21 days; (iii) group C_{1-21}/OL_{22-42} included rats to which olive oil intake was initiated from the 22nd day and lasted until the 42nd day; (iv) group Cd_{1-42} included rats that received intraperitoneally (i.p.) 1 mg of $CdCl_2$/kg/day for 42 days; (v) group DSF_{1-21} included rats that received *per os* 178.5 mg of DSF/kg bw/day for 21 days; (vi) group Cd_{1-42}/DSF_{22-42} included rats that were continuously treated with $CdCl_2$ and DSF, which was introduced on day 22, with treatment lasting until day 42 of the experiment. After the treatments, the animals were anesthetized with sodium pentobarbital (50 mg/kg) via injection, followed by decapitation. The testes were removed immediately, weighed and stored at -80°C.

Biochemical analysis of oxidative stress

Parameters of the oxidative status [$O_2^{\bullet-}$, MDA, glutathione (GSH), glutathione disulfide (GSSG), activities of tSOD, CAT, GR and tGST] in testicular tissue, as well as testicular and plasma testosterone concentrations were examined. About 100 mg of testes tissue were transferred into 1 mL ice-cold buffered sucrose (0.25 mol/L sucrose, 0.1 mmol/L EDTA in sodium-potassium phosphate buffer, pH 7.2) to obtain a 10% solution, and homogenized twice with a Teflon pestle at 800 rpm for 15 min at 4°C (Tehnica Zelezniki Manufacturing, Slovenia). The homogenates were centrifuged at 1580 g for 30 min at 4°C. Supernatants were sonicated with three cycles (30 s sonication, 15 s pause) and used for the analysis of OS markers and protein measurements, or stored at -80°C until analysis. The samples were kept on ice because of instability of the analytes.

Glutathione determination

About 100 mg of testes tissue were transferred into 1 mL ice-cold saline solution followed by the addition of 1 mL ice-cold metaphosphoric acid (MPA) (5% w/v) for deproteinization. Homogenization was performed twice with a Teflon pestle at 800 rpm for 15 min at 4°C. The homogenates were immediately centrifuged at 5056 g for 25 min at 4°C. The supernatants were used for chromatographic analysis or stored at -20°C until analysis.

Superoxide anion radical assay

Quantification of $O_2^{\bullet-}$ was based on nitroblue tetrazolium (NBT) reduction to yellow-colored monoformazan by $O_2^{\bullet-}$, the absorbance of which was measured at 550 nm [11]. The results are expressed as nmol reduced NBT/mg proteins.

Superoxide dismutase assay

tSOD activity was determined indirectly by measuring the absorbance of the produced red-colored complex (formazan dye) between 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium (INT) and $O_2^{\bullet-}$ (substrate for SOD) at 505 nm, using a commercial SOD kit (Randox Laboratories, UK), based on previously elucidated principles [12]. With the increase of tSOD activity, a decrease of $O_2^{\bullet-}$ occurs and thus formation of the colored complex. The results are expressed as units of tSOD/mg proteins.

Catalase assay

CAT activity was assayed according to the described procedure [13]. The rate of hydrogen peroxide (H_2O_2) decomposition by CAT is proportional to the reduction of the absorbance at λ=240 nm. Results are expressed as units of CAT/mg proteins.

Glutathione-S-transferase assay

The activity of tGST was assayed spectrophotometrically [14]. The underlying principle of the method is the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST. The absorption maximum of the formed GS-DNB conjugate occurs at 340 nm. The results are expressed as units of tGST/mg proteins.

Glutathione reductase assay

GR was assayed indirectly using a commercial kit (Sigma Aldrich, USA) that utilizes the principles of the Ellman's method for colorimetric measurement of GSH [15]. Generated from the reduction of GSSG by the GR, GSH reacts with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) forming a yellow product 5-thio-2-nitrobenzoic acid (TNB) and GS-TNB. The rate of absorbance change at 412 nm is directly proportional to GR activity. The results are expressed as units of GR/mg proteins.

Malondialdehyde assay

MDA was measured spectrophotometrically [16]. Briefly, TBA reacts with MDA generating a fluorescent red complex with a measurable absorbance at 532 nm. The results are expressed as nmol MDA/mg proteins.

HPLC-UV determination of reduced and oxidized glutathione

Both GSH and GSSG were simultaneously measured using a modified high-performance liquid chromatography (HPLC-UV) method [17]. Isocratic chromatographic separation was carried out on a ZORBAX Eclipse AAA (4.6 x150 mm, 3.5 μm) analytical column (Agilent Technologies, USA.) at a flow rate of 1 mL/min at 40°C and UV detection at 215 nm. The mobile phase consisted of a 100-mM sodium perchlorate solution (pH 2.8 adjusted with 0.1% orthophosphoric acid). The results are expressed as nmol GSH or GSSG/mg proteins and as the ratio GSSG/GSH.

Testosterone assay

The electrochemiluminescent method was used for testosterone measurement in homogenates of testicular tissue and plasma. A commercial immunoassay kit "ECLIA" (Roche, Germany) was used for measurement of testosterone-binding globulin in plasma by a Cobas E 411 immunoassay analyzer, as described [18].

Protein determination

The Lowry method was used for protein measurement in tissue homogenates [19].

Histopathological analysis

The testes were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4 for 12 h at 4°C and transferred to graded sucrose (10-30% in 0.1 M PBS, pH 7.4) and frozen in 2-methyl butane. The tissue was kept at -80°C until sectioning on a cryotome. Cross-sections of 12 μm were placed on SuperFrost glass slides, dried for 2 h at room temperature, stained with hematoxylin and eosin (H&E) and fixed with DPX mounting medium (Sigma-Aldrich, USA) [20]. Images were acquired using an Olympus microscope (Olympus, Tokyo, Japan) bright-light microscope (10-20 x magnification). The morphological features of the stained sections were identified using digital images.

Statistical analysis

One-way ANOVA and Tukey's *post hoc* multiple tests were used (software GraphPad Prism, version 5.01) for statistical data analysis. Values are presented as the means±standard deviation. Differences were considered statistically significant for $p<0.05$.

RESULTS

Changes in oxidative status were estimated by both enzymatic and nonenzymatic assays (Fig. 1A-I). Subchronic exposure to Cd significantly increased $O_2^{\bullet-}$, MDA and GSSG concentrations and decreased GSH in the testes of treated rats (Fig. 1A, B, G, H). Cd increased the GSSG/GSH ratio (Fig. 1I). The activities of all measured enzymes (tSOD, CAT, tGST and GR) were reduced in the testes of rats treated with Cd for 42 days compared to the controls (Fig. 1C-F).

Administration of DSF provoked a significant increase in $O_2^{\bullet-}$ (Fig. 1A) and a decrease in MDA (Fig. 1B) concentrations. After 21 days of DSF treatment, the activities of tSOD and tGST were reduced as compared to the controls (Fig. 1C,E).

Parallel exposure to DSF and Cd significantly decreased testicular $O_2^{\bullet-}$ and MDA concentrations ($p<0.001$), respectively, compared to rats exposed only to Cd for 42 days (Fig. 1A, B). Coexposure to DSF with Cd restored the activities of testicular CAT and GR compared to the control values (Fig. 1D, F) and increased testicular tSOD and tGST activities as compared to the Cd_{1-42} group (Fig. 1C, E). The addition of DSF significantly decreased the GSSG/GSH ratio ($p<0.001$) compared to the Cd_{1-42} group (Fig. 1I).

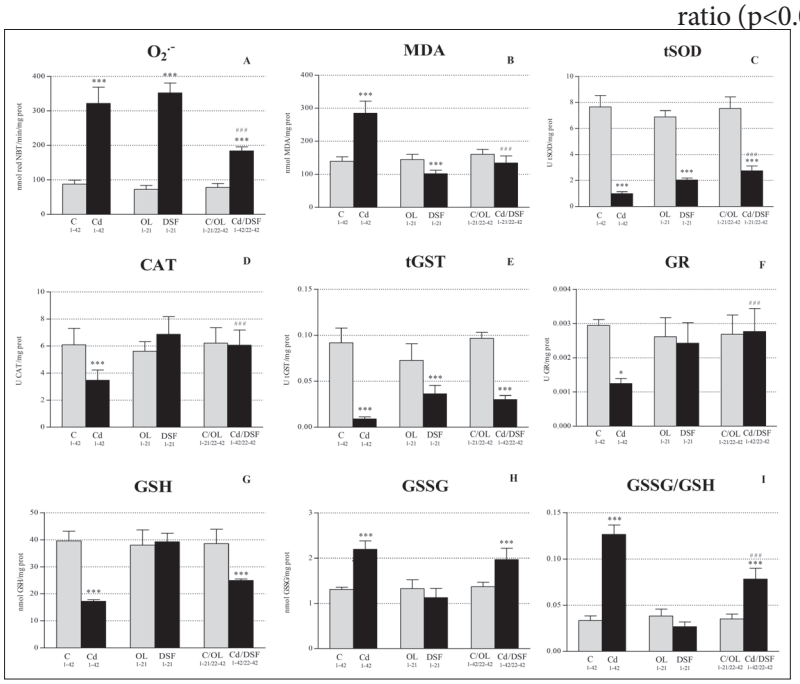

Fig. 1. The effect of disulfiram (DSF) on the oxidative status in testes of Wistar rats exposed to Cd. Animals received 1 mg of $CdCl_2$/kg/day *i.p.* for 42 days (Cd_{1-21} group), 178.5 mg DSF/kg BW/day *per os* for 21 days (DSF_{1-21} group) and $CdCl_2$ for 42 days with introduction of DSF from the 22-42nd day (Cd_{1-42}/DSF_{22-42} group). **A** – superoxide anion radical ($O_2^{\bullet-}$: nmol red. NBT/min/mg proteins); **B** – malondialdehyde (MDA: nmol MDA/mg proteins); **C** – total superoxide dismutase (tSOD: U tSOD/mg proteins), **D** – catalase (CAT: U CAT/mg proteins); **E** – glutathione-S-transferase (tGST: U tGST/mg proteins); **F** – glutathione reductase (GR: U GR/mg proteins); **G** – glutathione (GSH: nmol GSH/mg proteins); **H** – glutathione disulfide (GSSG: nmol GSSG/mg proteins); **I** – GSSG/GSH ratio. Values are presented as means±SD, for 6 animals/group. Differences were considered at three levels of statistical significance: $p<0.05$, $p<0.01$ and $p<0.001$. Legend: * – compared to the C group; # – compared to the Cd_{1-42} group. One Way ANOVA and Tukey's post hoc multiple tests were used for statistical analysis.

A significant decrease was observed in the plasma and testicular testosterone levels in both Cd_{1-42} and Cd_{1-42}/DSF_{22-42} groups in comparison to the control values (Table 1). Histological examination of tissue sections confirmed severe morphological changes in the testes of rats treated with $CdCl_2$ for 42 days. Cd induced the degeneration of seminiferous tubules and widening of the interstitial spaces (Fig. 2B). The normal architecture of testicular seminiferous tubules and interstitial spaces was confirmed by microscopic analysis of tissue sections of the DSF group. Seminiferous tubules were lined by stratified layers of cells on different stages of maturation (Fig. 2D). In testicular tissue after coexposure to Cd and DSF, a greater number of seminiferous tubules with thickened basal membranes were observed. The lumen of the tubules was almost completely acellular and filled with clod-homogeneous or crumbly-loose highly eosinophilic content. Some tubules were completely empty. The absence of spermatogenesis after coexposure to both agents was obvious (Fig. 2F).

Table 1. Effects of exposure of male Wistar rats to Cd and/or DSF on testicular and plasma testosterone levels.

Treatments	Testosterone in plasma (nmol/mL)	Testosterone in testes (ng/mg proteins)
C_{1-42}	20.00±2.217	5.91±1.014
OL_{1-21}	17.46±1.310	5.51±0.879
C_{1-21}/OL_{22-42}	21.36±2.164	5.65±1.089
Cd_{1-42}	5.62±0.971 ***	0.98±0.255 ***
DSF_{1-21}	16.86±2.590	4.85±0.067
Cd_{1-42}/DSF_{22-42}	0.868±0.772 ***	0.52 ±0.098 ***

Testosterone concentration were measured in the control group (C_{1-42}), disulfiram group (DSF_{1-21}), cadmium group (Cd_{1-42}) and Cd/DSF group (Cd_{1-42}/DSF_{22-42}). Values are presented as the means±SD. Differences were considered statistically significant for $p<0.05$. Legend: *** ($p<0.001$).

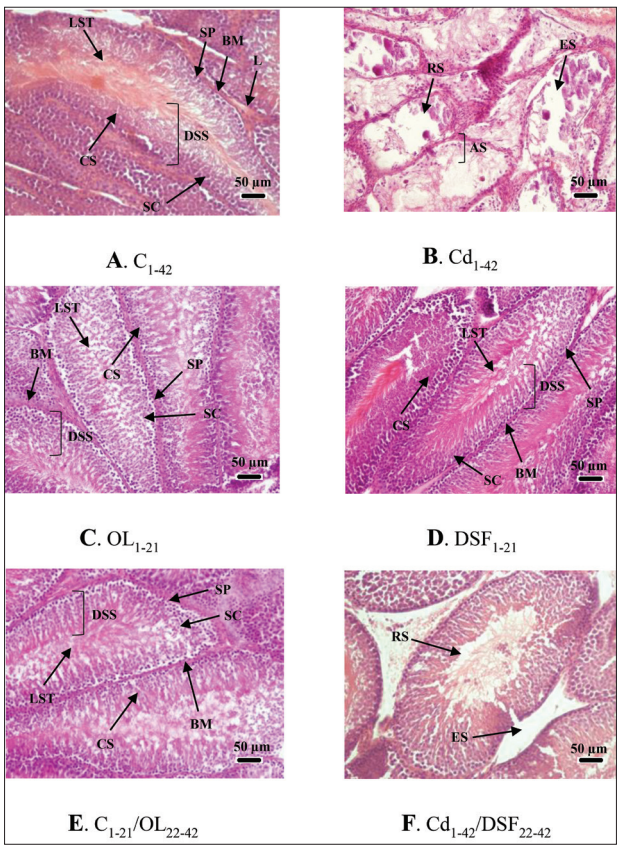

Fig. 2. Sections of testicular tissue after exposure to Cd and/or DSF vs matching controls. Animals received 1 mg of $CdCl_2$/kg/day *i.p.* for 42 days (Cd_{1-21} group) (**B**); 178.5 mg DSF/kgBW/day *per os* for 21 days (DSF_{1-21} group) (**D**); 1 mg $CdCl_2$/kg/day *i.p.* for 42 days with introduction of 178.5 mg DSF/kg BW/day *per os* from 22 – 42nd day (Cd_{1-42}/DSF_{22-42} group) (**F**). Tissue sections of controls: not treated rats (C_{1-42} group) (**A**); rats that received olive oil (solvent for DSF) for 21 days (OL_{1-21} group) (**C**); the rats that started with olive oil intake from 22 – 42nd day (C_{1-21}/OL_{22-42} group) (**E**). LST – lumen of seminiferous tubule, BM – basement membrane, SP – spermatogonium, SC – spermatocyte, CS – cytoplasm of Sertoli cell, L – Leydig cell, DSS – different stages of sperm development, AS – absence of spermatogenesis, RS – reduction of sperm cells, ES – edematous space.

DISCUSSION

Many studies of Cd toxicity and subsequent male reproductive failure have pointed to OS as an important cause of pathological changes [21,22]. Herein we confirmed that the OS-associated mechanisms of Cd reproductive toxicity modulate the functioning of biomolecules, especially lipids [4-6, 23]. Elevated levels of MDA (terminal product of LPO) in testicular tissue indicate deteriorated cell membrane function and permeability as a result of subchronic exposure to Cd [21,22]. Also, we showed that DSF coadministrated with Cd reduced ROS production and decreased LPO caused by Cd, thereby modulating the antioxidant response of testicular cells.

It is well known that the antioxidant enzymes CAT and SOD are essential for maintaining ROS homeostasis. CAT and SOD catalyze the biotransformation (sequestration) of hydrogen peroxide (H_2O_2) and $O_2^{\bullet-}$, respectively [24,25]. Sources of $O_2^{\bullet-}$ generation varied from endogenous (produced by xanthine and NADPH oxidases, byproducts of the mitochondrial respiratory electron transport chain, inflammation) to exogenous factors, including xenobiotic metabolism, toxic effects of some agents, etc. Although $O_2^{\bullet-}$ is not sufficiently potent to initiate LPO (unless it is protonated), it can oxidize low-molecular-weight reductants and react with nitrous oxide (NO) to generate the highly potent oxidant, peroxynitrite [26]. Additionally, H_2O_2 can undergo homolytic splitting to form the hydroxyl radical ($HO^{\bullet}$) or to participate in Fenton-like reactions together with transition metals to produce $HO^{\bullet}$. Cd affects both antioxidant enzymes. It binds to their -SH groups, displacing metals (cofactors) from their active sites, altering the secondary structure of CAT and SOD, which leads to enzyme misfolding and prevention of the access of substrate to the active site of the enzyme, thus reducing or inactivating their enzymatic activities [26-29].

DSF-like thiols of low molecular weight (such as D-penicillamine, N-acetylcysteine, captopril, tetrathiomolybdate) demonstrate both antioxidant and prooxidant effects [30, 31]. Therefore, agents that can reduce or increase oxidative conditions, such as DSF or DSF-like thiols, have been considered as OS modulators [32]. DSF metabolites have the potential to bind toxic Cd ions and/or other transition metals, which represents the platform for the observed antioxidant role of DSF during parallel exposure with Cd, which lessened its toxicity.

Two underlying mechanisms of the nucleophilic sulfhydryl (-SH) group of DDTC, the main metabolite of DSF, were responsible for the antioxidant role of DSF: reduction of FR (or FR scavenging), similar to GSH, and binding of transition metals (such as Cu), thus preventing the production of hydroxyl radicals via the Fenton reaction [30,33-35]. From day 22-42 of exposure to Cd, DSF exhibited a restoring effect on all measured parameters of OS in rat testes. The activities of testicular tSOD, CAT and GR were significantly higher in the Cd_{1-42}/DSF_{22-42} group compared to the Cd_{1-42} group. Coexposure to DSF and Cd caused a significant decrease in testicular $O_2^{\bullet -}$ and MDA levels compared to the rats that received only Cd for 42 days.

The glutathione redox cycle merges GSH and GSH-associated enzymes and has a crucial antioxidative defense role in the body. Glutathione scavenges FRs through nonenzymatic and/or enzymatically catalyzed reactions when it becomes converted immediately into its disulfide form, GSSG, which becomes efficiently reduced back to GSH by GR. GR normally maintains the total glutathione pool in a predominantly reduced state [36,37]. Additionally, GSH is involved in the conjugation metabolic reactions of xenobiotics (in metabolic phase II, the nucleophilic sulfur atom of GSH reacts with the electrophilic centers of xenobiotics). These conjugation reactions are catalyzed with GST. According to our results, prolonged exposure of rats to Cd significantly reduced the activities of testicular GR, tGST and GSH (which is also supported by the noted high GSSG/GSH ratio). Administration of DSF affected tGST activity [31]. However, tGST activity can be restored by an excess of GSH [38]. We showed that DSF restored GSH homeostasis that was negatively affected by Cd. Testicular GSH and GSSG levels in the Cd_{1-42}/DSF_{22-42} group did not significantly differ from the Cd_{1-42} group, unlike the GSSG/GSH ratio, which was significantly lower in the Cd_{1-42}/DSF_{22-42} group. Also, we showed that DSF introduced on the 22nd day of Cd exposure was not capable of increasing testosterone to the normal level until the 42nd day during the parallel exposure.

It appears that the DSF/DDTC redox couple acted as a biologically active redox GSH/GSSG couple. The redox couple DSF/DDTC could play different roles in various conditions, including modification of GSH level or scavenging ROS in a way similar to GSH. This scenario could explain the modulatory effect of DSF on the deteriorated OS in the testes of rats previously exposed to Cd [39,40].

The interstitial spaces of testes where Leydig cells are localized become edematous in response to Cd toxicity, which affects testosterone production as we previously reported [6]. Also, the observed histopathological alterations of testicular tissue, such as degeneration of seminiferous tubules, germinal cells and absence of spermatogenesis in the Cd group, appear to be irreversible. DSF could not improve testicular damage induced by Cd and *per se* it did not affect the morphology of testicular tissue, while tissue structure remained intact and sperm cells in all stadiums of spermatogenesis could be observed.

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