

Sublethal Effects of Cadmium Chloride to Testis of Zebrafish (*Danio rerio*)

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Abstract

Cadmium (Cd) is one of the most toxic environmental heavy metals to organisms. The prominent toxic effects of Cd on reproductive organs are very well known. Gonad histopathology is a valuable tool for the assessment of endocrine disruption compounds EDCs effects on fish. The aim of this study was to evaluate the reproductive toxicity of cadmium chloride (CdCl₂) on histopathology of Zebrafish (*Danio rerio*) testis under sub-lethal conditions. Male Zebrafish were exposed to four concentrations of CdCl₂ (0.002, 0.02, 2, and 20 mg/L) for 21 days in semi-static conditions. Histopathological changes in testis and Gonad Somatic Index (GSI) were studied. Treatment at 0.002 to 20 mg/L cadmium had no significant effect on the survival and condition factor. GSI decreased in a dose-dependent manner and the decrease was significant ($P < 0.001$) in the group that received the highest dose. Testis histological alteration consisted of reduced testicular size, blood–testis barrier (BTB) disruption, germ cell loss, testicular edema, hemorrhage, blood cell leakage, interstitial fibrosis, reduced sperm count, degenerated seminiferous tubules and abnormal Leydig cells. These data show that, Cd-induced treatment has an adverse effect on GSI decrease and can disrupt the histology of reproductive target tissues, highlighting the disruption of the blood–testis barrier (BTB), which is a major target of Cd toxicity in testis. Even a low dose Cd pollution may become a problem in the future, because Cd accumulates in the male reproductive organs and disturbs spermatogenesis and population stability.

Keywords: *CdCl₂, Histopathology, Blood–Testis Barrier (BTB), Reproductive, Toxicity*

1. Introduction

The endocrine disruption compounds (ECDs) are a large group of chemicals discharged into the aquatic environment from manufacturing of various industrial and consumer products (Mills & Chichester, 2005). Cadmium (Cd) is a widely spread toxicant with

endocrine disrupting properties and one of the most toxic industrial and environmental heavy metals known to damage renal, hepatic, respiratory and reproductive systems (Foote, 1999; Bertin & Averbeck, 2006). The prominent toxic effects of Cd on reproductive organs were recognized early on (Foster & Cameron, 1963). Various mechanisms have been suggested to explain Cd induced toxicity. Reactive oxygen species enhance

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lipid peroxidation, altered antioxidant system, DNA damage, altered gene expression and apoptosis (Nordberg, 2009; Siu et al., 2009). Numerous effects of Cd on reproductive endocrinology have recently been described, but the specific mechanisms underlying its degree of toxicity remains unclear (Kumar & Singh., 2010). A number of studies have also indicated the consequence of Cd exposure on the reproductive function of fish. Studies have shown that exposure to Cd damages gonads and impairs gametogenesis (Foote, 1999; Bertin & Averbek., 2006; Eidem et al., 2006).

The Zebrafish (*Danio rerio*) is increasingly used as an ecological model species for studies of stress physiology including the effects of the exposure to different environmental chemicals (Segner, 2009). In fact, Zebrafish has been found useful in EDCs screening, EDCs effects assessment and studying targets and mechanisms of EDCs action. So, Zebrafish have often been adopted as a standard species for toxicity testing (Yang et al., 2009).

The toxicity tests are necessary for water pollution evaluation because chemical and physical measurements alone are not sufficient to assess potential effects on aquatic biota (Abou El-Naga et al., 2005). Among the indicators of primary destruction potentially induced by toxins, histological alterations have recently been reported to be relevant endpoints. Histological biomarkers are indeed valuable indicators of the general health of fish and mirror the effects of exposure to a variety of anthropogenic pollutants (Barillet et al., 2010).

Considerable interest has been shown in recent years in the histopathological study while conducting sub-lethal tests in fish. Tissue variations in organisms exposed to a sub-lethal concentration of toxic pollutants are a functional response of organisms which provide information on the nature of the toxicant. Due to their key role in reproductive function, histopathological impairments of the gonads could have severe consequences on an entire fish population within a contaminated environment (Thompson & Bannigan., 2008). Recent studies have also associated reduced

male fertility, such as reduced sperm count and poor semen quality, in men exposed to Cd (Gill & College., 1983; Nordberg, 2009; Chouchene et al., 2011). These connection studies are critical since they represent the vulnerability of the testes to Cd toxicity, however, the underlying mechanism(s) was not known at the time. It should be noted that in vivo study of cadmium on Zebrafish testis, is lacking in the literature.

Also, gonad histopathology is a valuable tool for the assessment of endocrine-disrupting effects on fish (Xingang 2007). The molecular events evoked by hormonally active agents have an effect on the levels of cell, tissue, and organ organisation and morphology, and the nature of the evoked effects is specific and depends on the hormonal system that has been disrupted (Gill & College., 1983). In addition, histopathological changes, specifically those in the gonads, can be predictive of the (reproductive) fitness of the specimen under study and thus, for the fitness of the population.

The present study was undertaken to obtain information regarding histopathological effects of cadmium chloride on GSI and testis histopathology in Zebrafish under sub-chronic exposure and laboratory conditions. Cadmium chloride toxicology in fish is indeed generally limited to acute lethality data. Better information of sublethal impacts of cadmium chloride exposure in aquatic organisms would, therefore, allow ecotoxicologists to establish regulatory standards or suggestions for cadmium in aquatic compartments.

2. Materials and Methods

2.1. Test Organisms

Adult male Zebrafish with an age of about 120 d (35.14 ± 5.23 g) and (38.24 ± 3.11 mm), were obtained from a local dealer and acclimated to laboratory conditions for 2 weeks before the experiment. During the acclimation and experiment phases, fish were given daily supplies of standard fish pellets (1% of their body mass per day) and inspections were

conducted twice a day to discard wounded, diseased and dead fish. Organisms were kept at a maximal density of 5 fish/L in tanks filled with 10l of a $27\pm 1^\circ\text{C}$ synthetic water equilibrated by air-bubbling on a 12-h light: 12-h dark photoperiod (OECD Guideline et al. 2011). Ion concentrations in the synthetic water were (mg/L): 6.26 K^+ , 11.5 Na^+ , 4.74 Mg^{2+} , 11.6 Ca^{2+} , 32.4 Cl^- , 31.0 NO_3^- , 9.61 SO_4^{2-} and 0.45 CO_3^{2-} . Ethical considerations and animal rights in this investigation were considered and the study was approved by the international Agency for Protection of Experimental Animals and by the Inhouse Animal Welfare Committee.

2.2. Experimental Design

Stock solutions of CdCl_2 in saline (referred to as Cd) were prepared (Sigma–Aldrich, St. Louis, USA) at concentration 100 mg/L. Four groups of fish were subjected to serial dilutions of the stock solution of CdCl_2 of 0.002, 0.02, 2 and 20 mg/L. In addition to natural controls (unexposed males), solvent control (ethanol) and a positive control group (17- β estradiol; 10 ng/L) were employed in the study. Each treatment was performed in two different tanks (20 fish each). The test was performed by the semi-static (renewal) method in which the exposure medium was exchanged every 24 h to maintain toxicant strength and level of dissolved oxygen as well as minimizing the level of ammonia excretion during this experiment. The water quality parameters of the diluting water used in the tests and determined by standard methods (Gordalla, 2011) were as presented in Table 1. The exposure period lasted 21 days.

Table 1: Water quality parameters of test condition

Parameters	Values
pH	6.58 ± 0.32
Temperature ($^\circ\text{C}$)	27.3 ± 1.3
Dissolved oxygen (mg L^{-1})	7.32 ± 1.04
Free carbon dioxide (mg L^{-1})	4.95 ± 0.08
Alkalinity (mg L^{-1})	32.8 ± 1.75
Hardness (mg L^{-1})	148.56 ± 12.65

2.3. Histological Preparation

On the last day of experimentation, fish were anesthetized on ice and testis were excised and weighed for gonadosomatic index (GSI) determination. The GSI was expressed in g per 100 g body weight. Histological examinations were performed as described by (Khodabandeh & Abtahi, 2006). For histological studies, gonads were immediately fixed in Bouin’s fluid for 24 h. Fixed testis were dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections were cut at $4\ \mu\text{m}$ and collected onto glass slides, stained with haematoxylin and eosin solution, and then examined under a light microscope. Gonads from six males were examined from each treatment.

2.4. Statistical Analysis

The statistical package SPSS, version 16, (Chicago, IL, USA) were used for data analysis.

To compare the GSI exposure to different concentrations of CdCl_2 , one-way analysis of variance (ANOVA) was used. Also, two- way ANOVA analysis was used for mean length and weight of different treatments. The data were tested for homogeneity of variances at a significance level of $P < 0.05$ and probability values of less than 0.05 were considered as statistically significant. Significant means were subjected to analysis by Duncan’s multiple range test ($P < 0.05$).

3. Results

No mortality was observed during the experimental period in control and treatment groups.

The mean length and weight and GSI of fish from the control groups and CdCl_2 are presented in Table 2. Statistical analysis by two- way ANOVA indicated that the mean weight and length of male Zebrafish were not significantly different in the various treatments.

Also, there were no treatment-related effects of

CdCl₂ on condition factor (k) in any of the groups. But, In the CdCl₂ treatment, GSI was significantly lower than that of the control groups.

Changes in GSI in adult male Zebrafish exposed to different doses of CdCl₂ (0.002, 0.02, 2 and 20 mg/L) has been shown in fig.1.

Fig. 1 shows the numerical means of GSI values. Results showed a significant difference between CdCl₂-treated samples and controls. As a result, the GSI of 20 and 2 mg/L CdCl₂ treated males were found to be significantly less than that of the control males. This strongly suggests that CdCl₂ inhibited the development of the Zebrafish testis.

The histopathological results of control and CdCl₂ exposed fish testis are displayed in Figure 2.

Testicular sections from the control group had no visible histopathological alterations and composed of normal, well-organized and uniform seminiferous tubules with complete spermatogenesis and normal interstitial connective tissue (Fig. 2, a). The arrangement of the seminiferous tubules were regular and the tubular walls were smooth. The sertoli cells were

sparsely distributed, among the spermatogenic cells (i.e. spermatogonia, spermatocytes, spermatids) at different stages of differentiation.

There were no obvious histopathological differences between the control and the CdCl₂ low dose groups (i.e. 0.002 mg/L CdCl₂ groups). However, different degrees of seminiferous tubular changes and leakage blood cells from middle to severe were observed in CdCl₂ treated groups.

However, Histological examination revealed marked hypospermatogenesis and a decrease of spermatogenic cells in the experimental group. Twenty one days exposure to CdCl₂ caused injuries that mainly included reduced testicular size, blood-testis barrier (BTB) disruption, germ cell loss, testicular edema, haemorrhage, leakage blood cell, interstitial fibrosis, reduced sperm count, degenerated seminiferous tubules and abnormal Leydig cells (Figure 2, d -f). These damage to fish testis increased with increasing concentrations of CdCl₂. The most severe injuries in Zebrafish testis were observed in 20 mg/L (Figure 2, g-i).

Table 2: Mean (\pm SD) length and weight and GSI following 21 days exposure of CdCl₂ in male Zebrafish to different treatments.

Mean (\pm SD)	Control	Solvent control	17- β estradiol	CdCl ₂
Length (mm)	38.24 \pm 3.11 ^{a*}	38 \pm 2.28 ^a	38.09 \pm 3.20 ^a	37.94 \pm 2.98 ^a
Weight (g)	35.14 \pm 5.23 ^a	34.90 \pm 4.02 ^a	35.82 \pm 4.42 ^a	36 \pm 3.16 ^a
GSI (%)	6.01 \pm 0.15 ^a	5.28 \pm 0.49 ^b	4.79 \pm 1.28 ^c	3.03 \pm 0.43 ^d

* Values in a column followed by different letters are significantly ($P < 0. 05$) different (Duncan's multiple range test).

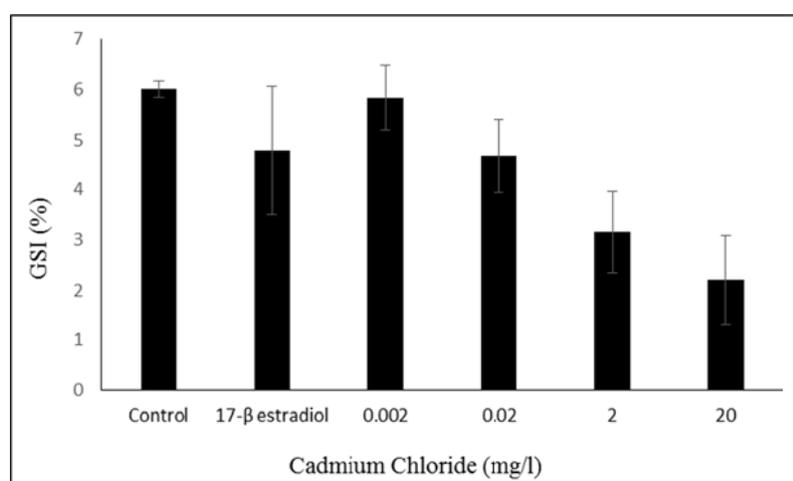


Fig. 1: Changes in the gonadosomatic index (GSI) in adult male zebrafish exposed to different CdCl₂. Data are given as mean (n=5) \pm standard deviation (SD)

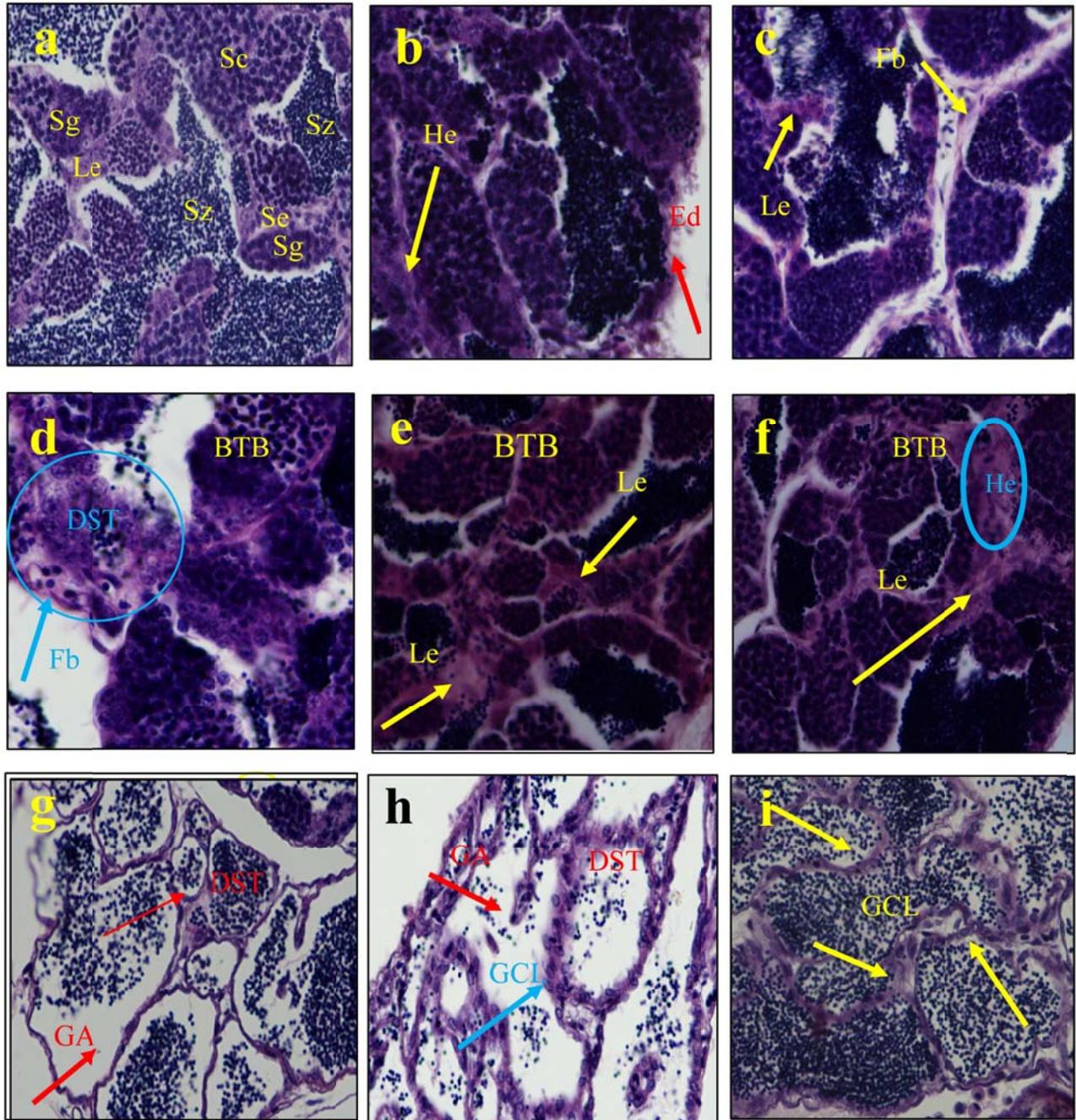


Fig. 2: Testicular histopathology in Zebrafish exposed to cadmium chloride for 21 days (H&E, 40X). The panels include a: control group, b-c: 0.02 mg/L, d-f: 2 mg/L, g-i: 20 mg/L. The testis of control fish indicated normal histology, while all treatment groups showed injuries that included blood–testis barrier (BTB) disruption; General atrophy (GA); blood cell Leakage (Le); Germ Cell Loss (GCL); Edemna (ED); Degenerated Seminiferous Tubules (DST); Hemorrhage (He); interstitial fibrosis (Fb). [Spermatogonia (Sg); spermatocyte (Sc); spermatid (St); spermatozoa (Sz); Leydig cell (Le); sertoli cell (Se)].

Also, slight distortion of seminiferous tubules was recorded at the CdCl₂ 20 mg/L dose group, and these changes were characterized by germ cells decrease, testicular edema and haemorrhage (Fig. 2). The increased inter tubular space, the engorgement of blood vessels, and the presence of an acidophilic

granular material in inter- tubular spaces.

In summary, after Cd treatment, disruption of the tight junctions in the microvessels led to the leakage of blood cells, most notably erythrocytes, into the interstitial space, causing hemorrhage and edema. Extensive inter tubular hemorrhages are observed as

well as a generalized disorganization of the inter tubular constituents. Within the seminiferous tubules the epithelium exhibits extensive areas of desquamation while the individual cells display pycnosis of nuclei.

4. Discussion

The results of our study clearly demonstrate that exposure of adult male Zebrafish to Cd negatively affects gonad histology as evidenced by an increased BTB disruption, germ cell loss, testicular edema, hemorrhage, blood cell leakage, degenerated seminiferous tubules and etc. These changes could indicate a disturbed reproductive output.

As for chronic Cd exposure, Cd was reported to cause hemorrhage in the capillaries of the testes, followed by degeneration and necrosis of the seminiferous tubules; these appearances resembled testicular infarction. The testes produce metallothionein 1 and 2, which actively incorporates zinc during spermatogenesis. Metallothionein also actively binds to Cd. Therefore, in the case of chronic Cd exposure, Cd accumulates in the testes (Xu et al., 2005).

The testis is extremely sensitive to Cd toxicity. Since the 1950s, studies have demonstrated that in vivo acute exposure to Cd caused BTB disruption, germ cell loss, testicular edema, hemorrhage, interstitial fibrosis and sterility in a few mammalian species (e.g., rodents, rabbit, dog, calf, stallion) (Bertin & Averbek., 2006; Siu et al., 2009; Barillet et al., 2010). The fish testis is the most important organ to encounter chemical pollutants, specially facing (EDCs) in the aquatic environments. The toxicant levels and pathological damage in testis reflect the pollution levels in the aquatic environments. Various components of Cd lethality have been recommended, including ionic and molecular mimicry, interference with cell adhesion and signaling, oxidative stress, apoptosis, genotoxicity and cell cycle disturbance (Thompson & Bannigan., 2008; Kumar & Singh., 2010).

As discussed above, the testis is extremely sensitive to Cd and one of the possible explanations

for this sensitivity is the remarkable morphological layout of the BTB. The blood–testis barrier is a physical barrier between the blood vessels and the seminiferous tubules of the animal testes. The name "blood-testis barrier" is misleading in that it is not a blood-organ barrier in a strict sense, yet is shaped between Sertoli cells of the seminiferous tubule and as such isolates the further developed phases of germ cells from the blood. A correct term is the "Sertoli cell barrier" (SCB) (Xu et al., 2005). These changes suggest a marked increase in the flow of fluid from the vascular bed of the testis to the extracellular spaces. These changes suggest a marked increase in the flow of fluid from the vascular bed of the testis to the extracellular spaces.

The term blood-testis barrier, otherwise called the Sertoli cell seminiferous epithelium barrier, in any case, was first used by Chiquoine (1964) (Chiquoine, 1964) in a study that examined the impacts of cadmium toxicity as it related to testicular necrosis. Other research showed that testicular morphology was greatly altered 3 months after initial Cd exposure, with degenerated seminiferous tubules, abnormal Leydig cells, fibrosis, and reduced the testicular size (Eidem et al., 2006). Initial studies that perceived that Cd could induce profound and irreversible harm to mammalian testes described the disruption of endothelial cells of microvessels, edema and hemorrhage by morphological examination (Parizek, 1964). The Cd-induced BTB damage has also been considered in vitro by using a model in which primary Sertoli cells isolated from 20-day-old rat (Janecki et al., 1992). Aoyagi et al (Aoyagi et al., 2002), produced compelling information demonstrating that Cd is indeed incorporated into early and late spermatids and spermatogonia. Disruption of the BTB by Cd, with consequent reduction in germ cell numbers and infertility, has been linked to reduced occludin expression.

Importantly, testosterone counteracted the Cd disruptive effects, potentially by inducing the expression of TJ integral membrane proteins such as occludin. Therefore, testosterone plays a crucial role

in the regulation of Sertoli cells TJ-permeability barrier, which is consistent with recent reports that androgen promotes the BTB integrity and cell adhesion function in the testis (Meng et al., 2005).

Cd is a known endocrine disruptor by affecting the synthesis and/or regulation of several hormones. Cd regulates androgen receptor (AR) gene expression and also mimics androgenic effects in orchidectomized rats and mice (Siu et al., 2009). These observations also illustrate that androgen (or a manipulation of the androgen receptor in Sertoli cells) can be a potential target candidate to manage Cd-induced testicular toxicity, which should be explored in future studies.

In male rodents, it is well established that Cd significantly alters the circulating levels of several hormones (e.g., testosterone, LH, FSH) (Parizek, 1964). Previous studies have shown that Cd impairs the testosterone production in isolated Leydig cells without affecting their viability, demonstrating that steroidogenic disruption in Leydig cells is likely to be an initial target of Cd toxicity as an endocrine modulator. Cd also decreased steroidogenic acute regulatory protein (StAR), LH receptor and cAMP levels in the testis (Meng et al., 2005; Janecki et al., 1992).

Several mechanisms have been proposed to mediate Cd-induced cellular toxicity. It has been proposed that Cd exerts its effects via the physicochemical properties of the Cd²⁺ ion, namely its similarities to Ca²⁺ (e.g., ionic radii) and Zn²⁺ (e.g., electron configuration). As such, Cd²⁺ is likely to substitute Ca²⁺ or Zn²⁺ in crucial physiological processes that are mediated by these ions, resulting in the activation and/or inhibition of several signalling pathways. For example, Cd may cause an increase in oxidative stress by binding to sulfhydryl groups of proteins and by depleting glutathione (Sangalang & O'Halloran., 1973). Our results suggest that the Cd-induced Zn depletion in testis tissues may be a causal factor in inducing testis damage in the Cd-exposed fish. In fact, Zn is a basic micronutrient that plays fundamental housekeeping roles in a variety of

biological activities for example growth and reproduction as well as in many cellular processes involving transcription, enzyme structure and activity, protein interactions, and even cell signaling (Chouchene et al., 2011). It is also involved in antioxidant defence. Free radicals and other reactive oxygen species (ROS) have been recently incriminated in the pathogenesis of various metals.

There are many reports proposing alterations in free radicals production and antioxidant defence system of the body after cadmium exposure (Kumar & Singh., 2010). The role of Cd in inducing oxidative stress in adult tissue has been well documented. Increased levels of malondialdehyde, protein oxidation and reduced activity of superoxide dismutase (SOD) in rat testes have been reported following a single SC injection of 2mg/kg CdCl₂ (Oteiza et al., 1999). The above discussion supports the argument for cadmium acting specifically upon the vascular system of the testis whose breakdown subsequently leads to an ischemic necrosis of the seminiferous tubules.

Conclusions

Cadmium enters into the aquatic ecosystem through anthropogenic activity and gets further biomagnified in the food chain. These results revealed the high concentration of Cadmium in testis, disrupts the normal male reproduction resulting in male sterility. These data show continuous exposure to subacute concentrations of cadmium for 3 w can cause testicular damages and unreturnable reproductive effects in male Zebrafish. This study indicates that high levels of Cd in the environment and its accumulation in fish are potential risk concerns of reproduction health. Cd has the potential to affect reproduction and development in many different ways at every stage of the reproductive process. The experimental model and approach used in our study provides a new and powerful tool for investigating the site and mechanism of action of known and potential cadmium which are suspected to

affect spermatogenesis by compromising the function of the blood-testis barrier.

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