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Cadmium as an etiology of sperm dysfunction in Holstein bulls

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Summary

Oxidative stress has been identified as a crucial factor leading to male infertility largely due to peroxidative damage to the sperm cell membrane. Cadmium (Cd) is a widespread environmental pollutant and one of the well-known reproductive toxicants. Because of its long biological half-life (10–30 years), Cd accumulates in the biological systems. The present study was designed to assess the concentration-dependent in vitro effect of 20 to 700 µM of Cd on the membrane integrity, motility, and acrosomal status of Holstein bull spermatozoa. We recorded a significant elevation in the malondialdehyde (MDA) level, increased lipid peroxidation (LPO) rate and a drastic decrease in the spermatocrit values, especially at the 700 µM concentration of Cd, indicating deleterious effects of Cd on the intactness of the sperm membrane. There was also a negative correlation between the LPO rate and both the percentage of motile spermatozoa (r = -0.89) and sperm viability (r = -0.86). Performing the gelatin test indicated that Cd altered the integrity of acrosomal membranes and showed an abnormal acrosome reaction. In this regard, a reverse correlation was found between the LPO rate and the percentage of halos (r = -0.96). In conclusion, Cd was proved to be a potential toxicant in the category of environmental factors that induced membrane impairments, lowered motility and viability, and decreased rate of acrosome reactions leading to bull sperm dysfunction.

Key words: Cadmium, Bull sperm, Membrane integrity, Motility, Acrosome

Introduction

Due to increased stress, lifestyle factors and a variety of industrial pollutants, the sperm quality and its fertilizing ability have decreased dramatically (Arabi *et al*., 2003). This may suggest that the quality of semen has deteriorated partly due to the effects of increasing environmental toxic factors. Environmental factors have been suggested to play a role in animal infertility (Hovatta *et al*., 1998). More importantly, environmental factors differ between areas with higher amounts of pollutants closer to industries (Benoff *et al*., 2000).

There is epidemiological evidences indicating that exposure to industrial metal aerosols may be detrimental to the male reproductive systems which is not reversible by short periods of non-exposure (Bonde, 1990). Metal ion contamination has also been associated with male reproductive toxicity in the experimental animals and may have the potential to produce adverse effects on fertility (Rao and Sharma, 2001).

Oxidative stress has been identified as a crucial factor leading to male infertility, largely due to peroxidative damage to the sperm cell membrane. Reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$) and superoxide anion ($^{\bullet}O_2^-$) are capable of adversely modifying cell function and ultimately endangering the survival of the cell. ROS-mediated lipid peroxidation (LPO) causes impairment of sperm function that is reflected in decreased pregnancy rates (Sharma and Agarwal, 1996; Agarwal *et al*., 2003). A shift to a more oxidative state may cause LPO, DNA damage, metabolic impairment, and inactivation of enzymes and membrane ion transporters in spermatozoa (Bilodeau *et al*., 2000; Agarwal *et al*., 2003; Arabi *et al*., 2003; Moustafa *et al*., 2004). It has been shown that heavy metal ions may produce LPO, which is associated with improper sperm motility (Suleiman *et al*., 1996).

The heavy metal cadmium (Cd) is

widely distributed in the environmental workplace (Friberg *et al*., 1986). Cd accumulates in biological systems and because of its long biological half-life (10–30 years), it accumulates in animals, as they grow old. This metal has adverse effects on the testes, sperm function and fertility in human and animals (Paufler and Foote, 1969; Benoff *et al*., 1997; Foote, 1999). Concentrations of Cd have been reported to be 0.8 and 1.2 µg/dl in human seminal plasma taken from normospermic and oligospermic men, respectively (Abou-Shakra *et al*., 1989). The flow of Cd in ecological systems increases, due to human contamination by major sources such as mining, smelting, cigarette smoking and industrial use (Jones and Cherian, 1990; Paksy *et al*., 1997; Gunnarsson *et al*., 2003). Cd promotes early oxidative conditions and contributes to the development of serious pathologic conditions because of its long retention time in some tissue (Bagchi *et al*., 2000). Cd can also induce apoptosis in rat germinal cells, in vitro (Xu *et al*., 1996). In human, the concentrations of Cd in blood or seminal plasma were found to be inversely correlated with sperm count and motility (Xu *et al*., 1993).

Cd preferentially accumulates in male reproductive organ (Gunnarsson *et al*., 2003). Cd is also capable of affecting male reproductive system via decreasing biosynthesis of LH receptor mRNA, testicular necrosis and decreased sperm motility rate in rats and rabbits (Parizek and Zahor, 1956; Young *et al*., 1995; Gunnarsson *et al*., 2003). An in vivo study demonstrated that Cd may reduce rat sperm movements at a dose far below the dose affecting sperm production (Xu *et al*., 2001). However, scarce information is available on the in vitro influence of Cd contamination on spermatozoa as a model for bull spermatozoa.

The objective of the present study was to investigate whether in vitro exposure to Cd ($CdCl_2$) would affect the membrane integrity, motility and acrosomal status of bull spermatozoa. The concentrations of Cd used in this study were not environmentally relevant but was constituted a model system to examine bull sperm exposure.

Materials and Methods

Nine semen samples were obtained from Holstein bulls (<20% abnormal sperm and >70% sperm motility) with normal (intact) acrosome. The pure ejaculates were collected from Jahad agriculture center, Kabootar Abad, Ziar area, Esfahan province in Iran. The semen samples were transported to the laboratory under 15°C and evaluated on the same day. Seminal plasma was then discarded by centrifuging the samples at 300 × g, for 10 min. The sperm pellet so obtained was suspended in 0.2 M phosphate buffered saline (PBS) (pH = 7.2; 1:5). One-tenth ml of this sperm suspension was used in each experiment. All chemicals were obtained in analytical grade from Sigma Chemical Co. (St. Louis, MO, USA), and working solutions were made in the degassed double-distilled water. In all tests, the incubation time was 60 min.

Cadmium concentration range

Cd as $CdCl_2$ was used at different concentrations (20, 100, 300, 500 and 700 µM, respectively). Throughout the study, 0.1 ml of each concentration was added to the sperm samples.

Experimental groups

The experimental groups were categorized into three groups; 1) negative control (intact), with spermatozoa but without Cd and hypo-osmotic solution; 2) positive control, with spermatozoa and hypo-osmotic solution but without Cd; and 3) treated samples, with spermatozoa and different concentrations of Cd.

Evaluation of membrane integrity

a- Lipid peroxidation (LPO) test

Thiobarbituric acid (TBA) reactivity was used as an index of LPO in the control and Cd-treated bull sperm samples by reacting malonyldialdehyde (MDA), a major by-product of LPO process and TBA to form MDA-TBA complex. Briefly, in the assay mixture, 0.1 ml of sperm sample plus adequate amounts of PBS (pH = 7.2) and 150 mM tris-HCl buffer were incubated at 37°C for 60 min. The reaction was stopped by adding 1 ml of chilled 10% TCA. Sperm

samples were then centrifuged (2500 × g, 10 min) and an aliquot of 1 ml was taken out into another set of tubes containing 1 ml of 2% TBA in 0.05 N NaOH. The tubes were mixed thoroughly and immersed in boiling water bath for 10 min and cooled at room temperature. The Cd-treated sperm samples and controls were then analysed for MDA levels with high-performance liquid chromatography (HPLC) separation of the MDA-TBA adduct using a Hitachi D6000 HPLC with an L-4200 UV-vis detector set at 532 nm (Wong *et al*., 1987). At the end of the LPO process, 25 µl of each sample (maintained at 4°C in the autosampler) was injected into the HPLC. Samples were eluted with 65% 50 mM KH_2PO_4-KOH (pH = 7.0) and 35% methanol at a flow rate of 1 ml/min with a µ-Bondapak C_{18} 3.9×300 mm Waters column. 1,1,3,3-tetra-ethoxypropane was used as the standard to generate a calibration curve for MDA.

b- Spermatocrit test

To evaluate the sperm functional integrity, the spermatocrit technique was used (Lagares *et al*., 1999), with some modifications (Arabi, 2004a). Briefly, the Cd-treated samples and negative control (untreated) groups were incubated at 37°C for 30 min, and then centrifuged at 300 × g, for 10 min. The sperm pellet was homogenized by mixing and incubated with a hypo-osmotic solution (150 mOsm sodium citrate + 150 mOsm fructose solution, 1:9) for 60 min at 37°C. The samples were then aspirated in the spermatocrit (haematocrit) tubes and centrifuged (15000 × g, 3 min) using a commercial haematocrit centrifuge set (micro-centrifuge K80 h, Wagtech, Birmingham, England).

Sperm viability test

To determine the percentage of live and dead sperm cells, a vital staining technique with eosin was used (Blom, 1950). A total 400 spermatozoa on a prepared slide was observed with a light microscope. The percentages of live (unstained) and dead (pinkish to red) spermatozoa were calculated in the treated and control groups.

Sperm motility test

To determine the percentage of motile and viable sperm cells, a drop of semen suspensions was placed on a slide, covered with a coverslip, and samples were assessed under a phase contrast microscopy. At least, 200 spermatozoa were examined per suspension droplet. The pattern of sperm movements was not evaluated (Verma and Kanwar, 1998).

Gelatin digestion test

The gelatin digestion test (acrosomal status) was performed to determine the ability of bull spermatozoa to undergo acrosomal exocytosis (Fiscor *et al*., 1983). Slides were cooled by keeping in a moist chamber at 4°C, for 2 hrs. One-hundred µl of 2.5% gelatin suspension (dissolved in boiling distilled water) was placed on a pre-cooled microscopic slide and a smear was made. Slides were then kept in horizontal position until dried, fixed for 2 min in 0.05% glutaraldehyde, thoroughly washed in PBS and then kept overnight in a moist chamber at 4°C. Fifty µl of sperm suspensions was placed on one end of the slide and smeared with a cover glass. Slides were placed in a horizontal position until dried and then incubated in a moist chamber at 39°C for 24 hrs. Slides were then stained with Comassie blue and examined with light microscopy (×400) for evidence of gelatin digestion. Sperm with a bright clear zone (halo) around the head were considered to have the ability to digest gelatin. The percent of halos was evaluated by measuring at least 400 sperm cells on one slide. Three slides were prepared for each treatment. The percentage of sperm with/without a halo was calculated for each slide.

Statistical analyses

Data was statistically analysed by paired Student's t-test (SPSS, version 11.0) to establish the validity of the investigation. All measurements were performed in the repeated triplicate. All the values were expressed as mean (±SD).

Results

To determine the possible deleterious effect of Cd on sperm membrane organization, LPO and spermatocrit tests were performed. The results from LPO analysis

showed that Cd at different concentrations (20–700 µM) elevated the MDA level/LPO rate in bull sperm suspensions, in a concentration-dependent manner by 3.32% ($P>0.05$), 19.09% ($P<0.05$), 26.77% ($P<0.01$), 36.52% ($P<0.01$) and 39.42% ($P<0.01$), respectively (Fig. 1). There was a positive correlation between the upgrade concentrations of Cd and the LPO rate ($r = 0.94$, $P<0.001$).

Fig. 1: Effect of different concentrations of Cd on the LPO rate in the bull sperm samples. [a]p<0.05 and [b]p<0.01: as compared to the negative control

As expected, the percentage of swollen spermatozoa, as standardized spermatocrits, in the positive control (with hypo-osmotic medium) group was high (61%, $P<0.01$) as compared to the negative control (without hypo-osmotic medium) group. Following incubation of positive control group with different concentrations of Cd, a decline in the number of swollen spermatozoa, as decreased spermatocrits, was recorded in a concentration-dependent manner. The highest value was obtained in the study group, which supplemented with 700 µM Cd by about -47.24% ($P<0.001$; Table 1). We observed a negative correlation between the decreased spermatocrit values and MDA level ($r = -0.82$, $P<0.01$).

With decreased membrane fluidity/ enhanced LPO, viability would be expected to decrease in some degrees. The analysis of results obtained from viability test showed that Cd at different concentrations induces a remarkable fall in the percentage of viable sperm cells (Table 1). We recorded a negative correlation ($r = -0.86$, $P<0.001$) between MDA level and the percentage of viable bull spermatozoa.

Table 1: Effect of different concentrations of Cd on the swelling reactions (spermatocrits), viability and acrosome reactions (% halos) in the normal bull sperm samples

Study groups	Spermatocrit	Sperm viability (%)	Halos (%)
Negative control	1.0	83 ± 3.8	73 ± 1.6
Positive control	1.60 ± 0.08[b]	81 ± 3.1	71 ± 1.01
20 µM cadmium	1.29 ± 0.08[*]	64 ± 2.7	66 ± 2.08
100 µM cadmium	1.14 ± 0.06[**]	55 ± 3.8[a]	55 ± 2.2[a]
300 µM cadmium	0.874 ± 0.07[***]	48 ± 2.4[b]	43 ± 1.9[b]
500 µM cadmium	0.856 ± 0.06[***]	46 ± 3.3[b]	42 ± 2.2[b]
700 µM cadmium	0.844 ± 0.07[***]	40 ± 3.01[c]	39 ± 2.6[c]

[a]p<0.05, [b]p<0.01 and [c]p<0.001: as compared to negative (without hypo-osmotic medium) control. [**]p<0.01 and [***]p<0.001: as compared to positive (with hypo-osmotic medium) control

The results showed the adverse effect of Cd on the sperm acrosome reaction. As described in Table 1, the halo (gelatin digestion regions) formation rate was decreased gradually from 73 ± 1.6 in negative control to 39 ± 2.6 in 700-µM Cd-treated group ($P<0.001$). The correlation coefficient between the percentage of halos and LPO rate was -0.96 ($P<0.001$).

Data from Fig. 2 also demonstrated that the proportion of spermatozoa with proper motility was decreased, with increasing the Cd concentration, from 83% in the negative control to 53% in 700-µM Cd-treated sperm samples ($P<0.01$). There was an inverse correlation ($r = -0.89$, $P<0.001$) between MDA level and the percentage of motile spermatozoa. In addition, the percentage of motile bull spermatozoa was positively correlated with spermatocrit ($r = 0.97$, $P<0.001$).

Discussion

Cd is widely distributed in the

Fig. 2: Effect of different concentrations of Cd on the bull sperm motility. [a]p<0.05 and [b]p<0.01: as compared to the negative control

environment (Foote, 1999). This metal is present at low concentrations in the semen of healthy presumably unexposed men (Noack-Fuller *et al*., 1993). Men working in industries with high exposure to Cd have been reported to have elevated blood levels of this metal (Working, 1988; Gennart *et al*., 1992).

In this study, the obtained data indicate that Cd was a potent oxidant when added to the bull sperm samples by increasing the MDA level/LPO rate. El-Demerdash *et al*., (2004) showed that treatment with Cd caused a significant increase in LPO rate and a decrease in sperm count and motility in rats. These authors suggested that Cd may replace calcium in calcium binding proteins causing disruption in their enzymatic activity, which can lead to oxidative stress. When Cd doses, exposure time, and temperature of incubation were increased, the production of LPO was also elevated (Yiin and Sheu, 1998). Metal ions as transition metals cause cellular damage via formation of highly reactive oxygen free radical viz. OH which is derived from O_2^- and hydrogen peroxide (H_2O_2) under Haber-Weiss reaction (Halliwel and Gutteridge, 1984).

One of the main reasons for LPO propagation after metal ion exposure is a disturbance in cellular glutathione content that may allow ROS to be free and attack double bonds in membrane lipids resulting in an increase in the LPO process (Arabi, 2004b). Shaikh *et al*., (1999) reported GSH depletion in animal tissues following treatment with Cd. Among phospholipids, phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) seem to be more susceptible to LPO-induced by metal ions (Delnomdedieu *et al*., 1992). The endpoint of LPO is the thiobarbituric acid-reacted MDA as LPO indicator which can cross-link between PS and PE, PS and PS, and PE and PE (Alvarez *et al*., 1987). Lack of uniformity in these cross-links in the membrane leads to a physical force that ultimately may disturb the membrane lipid distributions.

Cd bore a reverse correlation with the number of swollen bull spermatozoa. In the present study, spermatocrit test was performed as an indirect measurement of functional integrity of sperm membrane after being exposed to different concentrations of Cd. The hypo-osmotic swelling (HOS) test is an easy assay to assess the intactness of sperm membrane in which an influx of water results in an expansion of spermatozoon volume (swollen cells). High percentage of swellings is equal to high rate of normal membrane integrity. In the present study, 60% increase in the spermatocrit values of positive control group was observed as compared to the negative control group. It could be explained by a large swelling reaction of spermatozoa due to osmotic challenge in the spermatocrit tubes. More increase in the Cd concentrations led to more decrease in the spermatocrit values indicating the deleterious effect of Cd on sperm membrane intactness towards eliminating the semi-permeability properties of sperm membrane barrier. It may suggest that Cd alters the organization of sperm membrane lipids resulting in defective spermatozoa. A maximal decrease in spermatocrit values was seen in the 700 µM Cd-treated bull sperm samples, probably because of the high percentage of damaged or dead spermatozoa, which had lost the ability to expand.

A remarkable drop in the number of unstained live cells in the Cd-treated bull sperm samples was indicative of an increase in the number of defected sperm cells under the Cd stress. Furthermore, a positive

correlation was seen between decreased spermatocrit values and eosin-staining results. It may be noted that the spermatocrit test is used as an indicator of the biochemical integrity, whereas the viability staining (eosin) indicates the physical integrity of the sperm membrane. Rao *et al*., (1989) found that LPO might slow sperm movement via impairing the plasma membrane ion exchanges. Oxidative stress-mediated damages to the sperm membrane may account for defective sperm function observed in a high proportion of infertility cases (Aitken, 1994; Sharma and Agarwal, 1996).

Release of sperm hydrolytic enzymes (acrosome reaction) is essential for zona penetration and fertilization. Based on reports, Cd could inhibit the hyperactivation of rabbit spermatozoa (Young *et al*., 1995). As the hyperactivated sperm cells present acrosome reaction and a new powerful swimming pattern to reach ovum, it is plausible that the fertilization rate could therefore be affected by addition of Cd to the spermatozoal suspensions. According to the current results, addition of Cd exerted a significant decrease in the percentage of halos on the gelatin slides. Functional defects caused by ROS and LPO are decreased or arrested motility, failed sperm-oocyte fusion, and abnormal acrosome reaction (Sharma and Agarwal, 1996).

However, in the present study, structural and functional alterations of the sperm membrane due to peroxidation conditions may be accounted for the absence of sufficient acrosome reaction in the Cd-treated sperm samples. We observed a negative correlation between the percent of halos in gelatin digestion test and MDA formation ($r = -0.96$). However, it is reasonable to suggest that Cd may cause a severe lack of fertility potential to the animal sperm cells via an impaired cell membrane and an abnormal pattern of enzymatic exocytosis during acrosome reaction.

LPO impairs plasma membrane ion exchanges, which is necessary for maintenance of sperm movements (Rao *et al*., 1989). Oxidative modification of Na^+/K^+-ATPase can eliminate the sperm motility (Woo *et al*., 2000). This biological function reveals a critical role for Na^+/K^+-ATPase in the sperm movement and its fertilizing ability. Sperm motility may account for an early and sensitive endpoint in assessment of Cd toxicity on male fertility. Xu *et al*., (2001) showed that Cd may reduce the motility of rat sperm at a dose far below than the dose affecting sperm production. Disruption in ATP supply through disorganization of mitochondrial membranes is a major cause of lowered sperm motility in the Cd-treated sperm samples (Au *et al*., 2000). Motility and O_2 uptake by ram sperm was inhibited by Cd administration (Alabi *et al*., 1985). The concentrations of Cd added in this study were higher than the concentrations found in the seminal plasma. It has been reported that 10 µM Cd affected the microfibrils in the bull flagellar apparatus (Kanous *et al*., 1993).

The conclusion drawn from this study is that Cd proved to be a potential toxicant in the category of environmental factors that induces membrane impairments, lowered sperm motility and viability, and decreased rate of acrosome reactions leading to sperm dysfunction. Apparently, the presence of Cd in the environment and seminal plasma exerts a toxic effect on spermatozoa. However, further studies are currently being performed in our laboratory to determine other aspects of Cd-mediated infertility in the domestic and non-domestic animal spermatozoa.

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