The Role of Mercury in the Etiology of Sperm Dysfunction in Holstein Bulls

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ABSTRACT : A large number of toxicological substances and pharmacological and physical agents can cause reproductive intervention at the cellular and molecular level. The present study was designed to assess the effect of mercury ($HgCl_2$) at 50 to 550 μM concentration ranges, *in vitro*, on the sperm membrane and DNA integrity, viability, and acrosomal status of normal bull spermatozoa. The samples were processed for sperm analyses using semen-diluting fluid (PBS, pH 7.2). We recorded a sharp increase in the lipid peroxidation/LPO rate; the highest was at 550 μM mercury concentration, indicating a deleterious effect of mercury on the sperm membrane intactness. There was also a strong negative correlation between LPO rate and % viable spermatozoa ($R = 0.987$, $p<0.001$). Data obtained from a comet assay technique revealed that mercury is capable of inducing DNA breaks in the sperm nuclei. Interestingly, 92% of DNA breaks were double-stranded. The correlation between LPO rate and % DNA breaks was 0.984. Performing the gelatin test indicates that mercury is able to alter the integrity of acrosomal membranes showing an abnormal acrosome reaction. In this regard, a strong link was found between LPO rate and % halos ($R = 0.990$, $p<0.001$). Collectively, mercury proved to be a potent oxidant in the category of environmental factors affecting bull spermatozoa. Hence, considering the wide spread use of mercury and its compounds, these metals should be regarded with more concern. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 3 : 335-340)***

Key Words : Mercury, Bull Sperm, Membrane and DNA Integrity, Viability, Acrosome Reaction

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). There is growing evidence to suggest that oxidative stress (OS) is involved in many aspects of male infertility. An imbalance between the production of reactive oxygen species (ROS) and their scavenging by antioxidants may results in the OS conditions (Sharma and Agarwal, 1996; O'Flaherty et al., 2003). Under certain circumstances, low levels of antioxidants in bovine spermatozoa are linked to the detrimental effects on sperm function (Armstrong et al., 1999). A shift to a more oxidative state may cause lipid peroxidation (LPO), DNA damage, membrane alterations, impairment in the metabolism, and inactivation of enzymes in spermatozoa (Agarwal et al., 2003; Arabi et al., 2003; Moustafa et al., 2004).

The major ROS such as hydroxyl radical ($^{\cdot}OH$), and superoxide anion ($^{\cdot}O_2^-$) are capable of adversely modifying cell function, ultimately endangering the survival of the cell. The main consequence of excessive ROS generation is the peroxidative damage to the sperm plasma membrane elements, which leads to an impairment of sperm function that is reflected in impaired *in vitro* fertilization and a decreased pregnancy rates (Agarwal et al., 2003). Spermatozoa are particularly very sensitive to ROS assault because of existence of high quantities of polyunsaturated fatty acids in their own membranes (Alvarez and Storey, 1995). Exposing spermatozoa to artificially produced ROS significantly increases DNA damage by modifying all bases and producing base-free sites, deletions, frame shifts, and DNA cross-links (Barroso et al., 2000; Duru et al., 2000).

Metal ion contamination, particularly mercury intoxication, has 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). Owing to the role of metal ions as important catalysts in living organisms finding the knowledge regarding to other aspects of these compounds seems much imperative. The potential health effects of mercury have been a matter of concern because of potential wide human exposure consequent to its wide spread use. Mercury is distributed throughout the environment from both natural sources (inorganic form) and human activities (organic form) and easily accumulated in the animal tissues. Most of the mercury in the atmosphere is elemental mercury vapor and inorganic form, which may be deposited in water, soil and sediments. Mercury vapor is highly lipophilic and is efficiently absorbed through the lungs and oral mucosa (Crinnion, 2000). Direct application of soil fertilizers and fungicides, leather tanning, wastewater treatment facilities, paper mills, disposal of solid wastes including batteries and thermometers to the landfills, are mainly sources of mercury in the environment which can affect the animal tissues (World health organization, 1989). However, insufficient information is available on the effect of mercury toxicity as an *in vitro* model for animal spermatozoa.

The present study was then aimed to assess the possible anti-fertility effect of mercury ($HgCl_2$) in a concentration range from 50 to 550 μM, *in vitro*, on bull sperm membrane

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and DNA integrity, viability, and acrosomal status. Concentrations of mercury used in this study were not environmentally relevant but do constitute a model system to examine bull sperm exposure.

MATERIALS AND METHODS

Subjects

We used semen samples from twelve Holstein bulls with less than 20% abnormal sperm and over 70% sperm motility and intact or normal acrosome. The pure ejaculates were collected from Jahad Keshavarzi center, Kabootar Abad, Ziar area, Esfahan Province in Iran. The semen samples were transported to our laboratory under 15°C, and tests performed on the same day. Seminal plasma then was discarded by centrifuging the samples at 300×g, for 10 min. The pellet so obtained was suspended in 0.2 M phosphate buffered saline (PBS, pH 7.2).

Chemicals

The used chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, and USA). All solutions were made in the degassed double-distilled water.

Lipid peroxidation test

In order to evaluate the lipid peroxidation (LPO) rate, the sperm samples were analyzed for malondialdehyde (MDA), a major by-product of LPO, by the thiobarbituric acid (TBA) reaction, with high-performance liquid chromatography (HPLC) separation of the MDA-TBA adduct at 532 nm (Hitachi D6000 HPLC, Tokyo, Japan, with an L-4200 UV-vis detector set) according to method described by Wong et al. (1987). In the end of LPO process, 25 µl of each sample (maintained at four °C in the autosampler) was injected into the HPLC set. 1,1,3,3-tetraethoxypropane (TEP) was used as the standard to generate a calibration curve for MDA. The rate of LPO was finally expressed as mM MDA/l.

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 (×40). The percentages of live (unstained) and dead (pinkish) spermatozoa in media at each of treatments as well as control groups were calculated.

Single cell gel electrophoresis (Comet) assay

The aim of this test was to detect the DNA strand breaks and alkali labile damages in the individual cells (Singh et al., 1988; Hughes et al., 1996). The DNA fragments so produced migrate towards the anode pole, at a rate inversely proportional to the size of the fragment during electrophoresis. The sperm cells were added to agarose-covered slides and allowed to solidify. The slides, then, put in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 10% DMSO, Triton-X-100), for one h at four °C to digest the nuclear and the cell walls and unfold DNA. Consequently, slides were put in the alkaline buffer for running the electrophoresis (15 min, at 0.862 V/cm^2). The slides finally stained with acridine orange, which bound to single-stranded DNA, fluoresces red and to double-stranded DNA fluoresces green and viewed under a fluorescent microscope (Excitation filter 515-560 nm; Dichroic filter 580 nm; and Suppresion filter 580 nm). The percentage of categories of DNA damages were scored as undamaged (no DNA migration), and damaged (migrated DNA) cells.

Gelatin digestion test

This test 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 four °C, for two h. Hundred microlitre of 2.5% gelatin suspension (dissolved in boiling distilled water) was placed on one end of a pre-cooled microscopic slide and smeared with another slide towards the other end. Slides were then kept in horizontal position until dry, fixed for two min in 0.05% glutaraldehyde, thoroughly washed in PBS, and then kept overnight in a moist chamber at four °C. Fifty µl of sperm suspensions was placed on one end of a slide and smeared with a cover glass. Slides were placed in a horizontal position until dry and then incubated in a moist chamber at 39°C for 24 h. 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 percentage of sperm with/without a halo was calculated for each slide.

Statistical analysis

The paired comparisons were conducted with Student's paired T-test (control against treatments) using SPSS software (version 11.0). All measurements were performed in the repeated triplicates. The regression analysis was also performed between parameters assayed in the current study. All the results were expressed as means±SD.

RESULTS

The analysis of results obtained from LPO test showed that mercury in the different concentrations (50, 150, 350 and 550 µM) induced a remarkable peroxidation to the sperm membranes, in a concentration-dependent manner by 15, 30.31, 41.90 and 50.72%, respectively (Table 1). There

Table 1. Effect of different concentrations of mercury on the lipid peroxidation (LPO) rate and viability of bull spermatozoa

Study groups	LPO rate	Viability (%)
Negative control	0.420±0.03	78±34.88
50 M mercury	0.483±0.04[a]	65±4.12
150 M mercury	0.559±0.03[b]	60±3.56[a]
350 M mercury	0.590±0.02[b]	60±4.11[a]
550 M mercury	0.633±0.02[c]	59±3.06[b]

[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$: as compared to the negative control.
Lipid peroxidation (LPO) expressed as mM MDA/l.

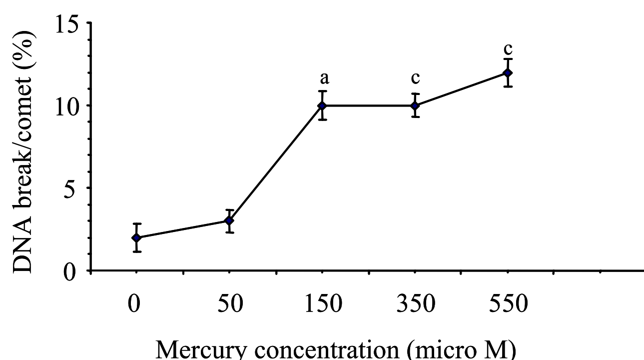

Figure 1. Effect of different concentrations of mercury on the DNA integrity (%comets/DNA breaks) of bull spermatozoa. (a) $p<0.05$, (c) $p<0.001$: as compared to negative control.

was a strong positive correlation between the upgrade concentrations of mercury and the LPO rate ($R = 0.953$, $p<0.001$).

Data from Table 1 show that the proportion of spermatozoa with intact membrane (viable cells) indicated by eosin staining, was decreased with increasing in the mercury concentration from 78% in the negative control to 57% ($p<0.01$) in 550 µM mercury-treated samples. We showed a negative link ($R = 0.987$, $p<0.001$) between LPO rate and % viable spermatozoa detected by eosin staining technique.

In Figure 1 and 2, the effect of mercury on DNA integrity of bull spermatozoa is also cited. Data obtained from comet assay technique show a linear increase in % DNA breaks (comets) from 2.0% (negative control) to 12.0% ($p<0.001$) in the 550 µM mercury-treated sperm samples. Interestingly, almost 92% of comets were of double-stranded DNA breaks with green fluoresces under acridine orange staining method. The positive correlation was between % comets and LPO rate values was 0.984 ($p<0.001$).

We showed also the influence of mercury on the bull sperm acrosomal status. In Figure 3, we demonstrated that percent of halos (gelatin digestion sites) was decreased gradually from 63±2.11 (negative control) to 44±2.04 (550 µM mercury concentration)($p<0.01$). The value of link between % halos and LPO rate was 0.990 ($p<0.001$).

Figure 2. Showing the effect of 550 µM mercury concentration on DNA integrity of bull spermatozoa detected by SCGE technique. Irregular forms are DNA breaks, and circular forms are normal nuclei without DNA breaks (×400).

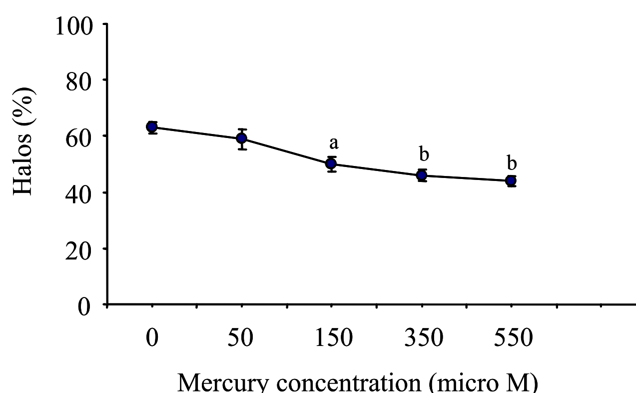

Figure 3. Effect of different concentrations of mercury on the acrosome reaction rate (% halos) of bull spermatozoa. (a) $p<0.05$, (b) $p<0.01$: as compared to negative control.

DISCUSSION

Environmental factors have been suggested to play a role in animal infertility (Hovatta et al., 1998). There is epidemiological evidence that exposure to industrial metal aerosols may be detrimental to the male reproductive systems and are not reversible by short periods of non-exposure (Bonde, 1990). Human civilization and a concomitant increase in industrial activity have gradually redistributed many toxic metals from the earth's crust to the environment. The potential health effects of mercury have been a matter of concern because of potential wide human exposure consequent to its wide spread use. However, at present, little is known of the exact anti-fertility mechanism of mercury effect on the sperm function in the animal models.

Mercury may induce oxidative damages in rat tissues as evidenced by increase in the MDA level and depleted GSH content (Sener et al., 2003). Of phospholipids, phosphatidyl

serine (PS) and phosphatidyl ethanolamine (PE) seemed to be more susceptible to the LPO induced by mercury. Mercury is capable of establishing bridges between three molecules of PS or of PE and altering their conformations (Delnomdedieu et al., 1992). The results obtained from our study also indicate that, mercury has an oxidant potential when added to the sperm samples in a concentration-dependent manner. Metal ions as transition metals cause cellular damages via formation of hydroxyl radical, which is derived from superoxide anion and hydrogen peroxide under Haber-Weiss reaction.

The end point of LPO process is the thiobarbituric acid-reacted MDA as an index of LPO damages, which can cross-link between PS and PE, PS and PS, and, PE and PE (Alvarez et al., 1987). Lack of the uniformity to these cross-links in the membrane will be led to a physical force, which may disturb the membrane lipid distributions. Here we showed a dramatic deleterious effect of mercury on the viability of spermatozoa. According to an investigation by Rao and Sharma (2001), mercury produced a significant reduction in the epididymal sperm viability and motility.

The earlier study from our laboratory showed that mercury additions to carp gill cells led to a markedly impaired GSH content, with an inverse proportion to oxidative condition, and that protection could be provided by supplementing media with antioxidants such as BSA and DMSO indicating a challenge between antioxidants and ROS-mediated LPO process (Arabi, 2004b). It seems that the metal ion extrusion from cells presumably involves movement of complexes such as Hg-GSH, which gives rise to alleviating in the level of cellular thiol pool including GSH in the sperm suspensions. Lee et al. (2001) demonstrated that the effect of mercury supplementation resulted in 34% reduction in GSH content, which was accompanied by 59% loss of cell viability. Our observations reveal that, mercury-induced loss of cell viability is accompanied by a significant elevation LPO rate. It may be assumed that mercury is able to provide free radicals, either by release of accumulated lipid hydroperoxides from sperm membranes or by direct generation of oxygen-derivatives, as a powerful fuel for peroxidation cascade resulting in the propagation of LPO process (De Flora et al., 1994). However, it needs further assessments.

A remarkable drop in the number of unstained live cells in the mercury-treated samples was indicative of an increase in the number of defected sperm cells under the mercury stress. LPO also impairs plasma membrane ion exchanges, which are for maintenance of sperm movement (Rao et al., 1989). 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).

Oxidative stress does not simply disrupt the fertilizing capacity of spermatozoa, it also attack the integrity of sperm chromatin and to cause high frequencies of single and double-stranded DNA breaks (Aitken and Krausz, 2001; Saleh et al., 2003). Exposing spermatozoa to artificially produced ROS significantly increases DNA damage by modifying all bases and producing base-free sites, defected frame shifts, and DNA cross links (Duru et al., 2000). Recent studies show a positive correlation between sperm DNA fragmentation and the levels of ROS in the testicular tissue (Rajesh et al., 2002) and in semen (Henkel et al., 2003). Human sperm samples exhibit a wide variation in DNA damages due to different contents of enzymatic and non-enzymatic antioxidant systems (Hughes et al., 1998). The data presented here provide evidence of an important relationship between mercury concentration effect and intactness of sperm membrane and DNA integrity. In the present study, an excellent positive correlation was found between increased DNA breaks and LPO rate values ($R = 0.984$), indicating that ROS-mediated LPO in the semen samples simultaneously causing DNA breaks in the sperm chromatin and oxidation in its DNA bases. Meanwhile, ROS modify lipid structure in the sperm membranes resulting in altered viability and movement variables. The $R = 0.887$ was calculated between % comets and sperm viability. The use of acridine orange (AO) stain helped us to distinguish single-stranded from double-stranded DNA breaks in sperm nucleus. Almost all the comets (92%) were of double-stranded DNA breaks. Reports indicate that sperm DNA integrity correlates strongly with male fertilizing ability *in vivo*, and infertile men have significantly poorer sperm DNA integrity and high levels of ROS than fertile controls (Fraga et al., 1996; Zini et al., 2001). Recently, we have also shown that nicotine-induced oxidative stress leads to extensive DNA damages in the human sperm suspensions (Arabi, 2004a).

Mercury is a reactive metal that has high affinity for macromolecules and binds to DNA leading to alteration in its structure (Ariaz and Williams, 1996). In addition, the inhibition of DNA repair processes may be an important mechanism in metal ion-induced genotoxicity, due to structural changes of DNA or modifications of repair proteins, or through out the competition with essential metal ions serving as co-factors (Hartwig, 1995). It has also been reported that mercury is capable of imposing a severe genotoxic effect in rat blood leukocytes (Grover et al., 2001). Our findings are consistent with the above observations.

A normal acrosome reaction (release of sperm hydrolytic enzymes) of spermatozoa is essential for zona penetration and fertilization. Our results show mercury exerts a meaningful reduction in the percent of halos on the gelatin slides. ROS mediated-functional defects caused lessened or arrested sperm motility, failed sperm-oocyte

fusion, and abnormal acrosome reaction (Sharma and Agarwal, 1996). However, here, structural and functional alterations of the sperm plasma membrane due to peroxidation condition may be accounted for the absence of sufficient acrosome reaction in the mercury-treated cells. However, it is reasonable to suggest that mercury may impose a severe lack of fertility potential to animal spermatozoa via impaired cell membrane and an abnormal pattern of acrosome enzymatic exocytosis.

Collectively, mercury proved to be a potential toxic agent in the category of environmental factors that induces membrane impairments, and DNA fragmentation leading to male infertility. Hence, considering the wide spread use of mercury and its compounds, these metals should regarded with more concern. However, further studies are currently being performed in our laboratory to determine other aspects of mercuric infertility in other domestic and non-domestic animal sperm samples.

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