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In vitro Mercury Exposure on Spermatozoa from Normospermic Individuals

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Abstract: Epidemiological evidence suggests that exposure to industrial metal aerosols is detrimental to the male reproductive system. Oxidative stress has been identified as a crucial factor leading to male factor infertility largely due to peroxidative damage to the sperm cell membrane. The objectives of the present study were to test the effect of mercury in the concentration range from 50 to 800 μmol^{-1}, *in vitro*, on the sperm membrane and DNA integrity, motility and acrosomal status of human spermatozoa. We found a significant increase in the Lipo Per Oxidation (LPO) indicating the deleterious effect of mercury on the sperm membrane integrity. This effect was prominent at the concentration of 800 μM mercury. There was also a strong negative correlation between LPO rate and percentage of viable spermatozoa ($r = -0.941$, $p<0.001$). Data obtained from SCGE assay technique revealed that mercury is capable of inducing DNA breaks in the sperm nuclei. Almost, 88% of DNA breaks were of double-stranded. The correlation between LPO rate and percentage of DNA breaks was found to be 0.918 ($p<0.001$). Performing the gelatin digestion test indicates that mercury was able to alter the integrity of acrosomal membranes showing an abnormal acrosome reaction. In this regard, a strong correlation was found between LPO rate and percentage of halos ($r = -0.893$, $p<0.001$). Taken together, mercury induced membrane impairments, lowered sperm viability, DNA breaks and a decreased rate in the acrosome reaction of human spermatozoa leading to sperm dysfunction. Entering mercury in the male gonads and seminal plasma may exert deleterious effects on the human spermatozoa. Hence, considering the wide spread use of mercury and its compounds, these metals should regarded with more concern.

Key words: Acrosome reaction, DNA-mercury, peroxidation, sperm cells, viability

INTRODUCTION

Mercury is a toxic metal widely spread in the environment with bio-accumulative features that raises public health concerns (Dorea and Donangelo, 2006). Male infertility is a main consequence of accumulation of such metals in the environment (Hovatta *et al*., 1998). Epidemiological evidence suggests that exposure to industrial metal aerosols is detrimental to the male reproductive system (Bonde, 1990). Metal ion contamination has also been associated with male reproductive toxicity in experimental animals and may have the potential to produce adverse effects on fertility (Arabi, 2005; Arabi, 2006; Rao and Sharma, 2001).

A large number of toxicological substances and pharmacological and physical agents can cause reproductive intervention of the cellular and molecular level. Oxidative stress (OS) is involved in many aspects of male infertility. An imbalance between the production of reactive oxygen species (ROS) such as hydroxyl radical (OH^{-1}) and superoxide anion (O_2^-) and their removal by antioxidants may result in the OS conditions (Sharma and Agarwal, 1996; O'Flaherty *et al*., 2003). A shift to a more oxidative state may cause Lipo Per Oxidation (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). 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)

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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 metal ion toxicity as an *in vitro* model for animal spermatozoa.

The present study was then aimed to assess the effect of mercury as $HgCl_2$ in concentration range from 50 to 800 μmol L^{-1}, *in vitro*, on the membrane and DNA integrity, viability and acrosomal status in the spermatozoa from the normospermic men. Concentrations of mercury used in this study were not environmentally relevant but do constitute a model system to examine human sperm exposure.

MATERIALS AND METHODS

Collection of sperm samples: We used semen samples from 30 young volunteers (28-30 years old), with proven normal sperm parameters according to World Health Organization (1999) criteria. After 30 min liquefaction at room temperature, seminal plasma then was discarded by centrifuging the samples at 500xg, for 10 min. The pellet so obtained was suspended in an equal volume of 0.2M phosphate buffered saline (PBS, pH 7.2) as semen diluting fluid.

Chemicals and incubation time: The used chemicals were obtained from Sigma Chemical Co. (St. Louis, MO and USA). All solutions were made in the degassed double-distilled water. In the following experiments, the incubation time was 120 min. at 37°C.

Evaluation of membrane integrity by lipoperoxidation (LPO) test: In order to evaluate the 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 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 1, 1, 3, 3-tetraethoxypropane was used as the standard to generate a calibration curve for MDA. The rate of LPO was finally expressed as mM MDA l^{-1}.

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 (x40). The percentage of live (unstained) and dead (stained) spermatozoa in media at each of treatments as well as control groups were calculated.

Single cell gel electrophoresis assay: This assay was performed according to a method by Hughes *et al.* (1996). The sperm cells (treated and control) 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 1 h at 4°C to 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 and viewed under a fluorescent microscope (Excitation filter 515-560 nm; Dichroic filter 580 nm and Suppresion filter 580 nm). DNA quality in human spermatozoa was evaluated by measuring at least 50 sperm cells on one slide (x400). Three slides were prepared for each treatment and control as well. Finally, the percentage of undamaged (without DNA breaks) and damaged (with DNA breaks) spermatozoa were recorded for each slide.

Gelatin digestion test: According to a method by Fiscor *et al.* (1983) a volume of 100 μL of 2.5% gelatin suspension was placed on one end of a pre-cooled microscopic slide and smeared with another slide towards the other end. Slides were dried and fixed for 2 min in 0.05% glutaraldehyde, thoroughly washed in PBS and then kept overnight in a moist chamber at 4°C. Sixty microliter of sperm samples were then smeared with a cover glass on a slide. 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 Coomassie blue and examined with light microscopy (x400) 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 (x400). Three slides were prepared for each treatment. The percentage of sperm with or without a halo was calculated for each slide.

Statistical analysis: The results were expressed in mean±SEM. The comparisons were conducted with Student's *t*-test when negative control and treated sperm

samples with mercury were compared (SPSS software, version 11.0. SPSS Inc., Chicago, IL, USA). The level of significance was set at $p<0.05$. All measurements were performed in repeated triplicates. Correlation analysis (Pearson's rank) was also evaluated between parameters assayed in the present study.

RESULTS

To evaluate the effect of mercury on the sperm membrane, the LPO test was performed. The analysis of results obtained from LPO test showed that mercury in the different concentrations (50, 100, 200, 400 and 800 μmol L^{-1}) induced a significant peroxidation to the human sperm membranes, in a concentration-dependent manner by 15.85, 35.61, 45.85, 57.32 and 66.59% (Fig. 1). There was a strong positive correlation between the upgrade concentrations of mercury and the LPO rate ($r = 0.932$, $p<0.001$).

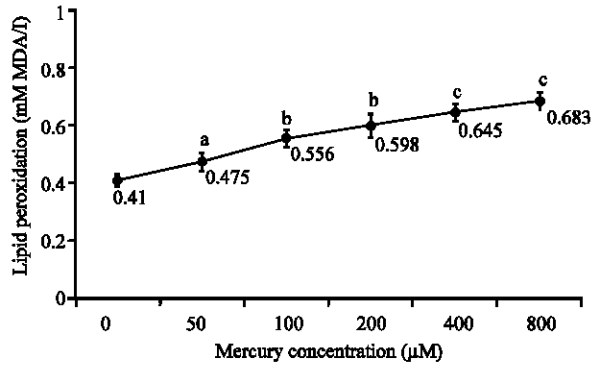

Fig. 1: Concentration effect of mercury on the lipid peroxidation rate in normal human sperm suspensions expressed as mM MDA/l. Data are expressed in mean+SEM, $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$: compared to the negative control

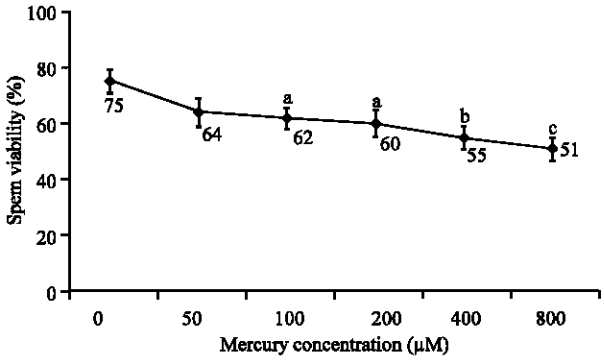

Fig. 2: Concentration effect of mercury on the cell viability in normal human sperm suspensions. Data are expressed in means±SEM, $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$: compared to the negative control

Table 1: Concentration effect of mercury on the DNA integrity and acrosome reaction rate of spermatozoa in the normospermic individuals

Study groups Concentration A mercury (μM)	DNA integrity (% DNA breaks)	Acrosome reaction rate (% halos)
Negative control	2.0±0.60	60±1.54
50	3.0±0.53	56±2.03
100	$10.0\pm0.61_{a}$	$53\pm1.42_{a}$
200	$11.0\pm0.42_{c}$	$50\pm1.72_{b}$
400	$11.0\pm0.53_{c}$	$46\pm1.55_{b}$
800	$12.0\pm0.58_{c}$	$44\pm1.61_{b}$

$^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$: as compared to the negative control. Data are expressed in mean±SEM

Data emerged from Fig. 2 show that the sperm viability was decreased with increasing in the mercury concentration from 75% in negative control to 51% ($p<0.01$) in the 800 μM mercury-treated sperm samples. There was a negative correlation ($r = -0.941$, $p<0.001$) between LPO rate and percentage of viable spermatozoa detected by eosin staining technique.

In Table 1, the effect of mercury on DNA integrity of human spermatozoa is also cited. Data obtained from DNA breaks assay technique show a linear increase in percentage of DNA breaks from 2.0% (negative control) to 12.0% ($p<0.001$) in 800 μM mercury-treated samples (Table 1). The $R = -0.871$ was calculated between percentage of DNA breaks and sperm viability. Interestingly, almost 88% of DNA breaks were of double-stranded with green fluoresces under acridine orange staining method. The positive value of correlation between percentage of DNA breaks and LPO rate values was 0.918 ($p<0.001$).

We showed also the influence of mercury on human sperm acrosomal status. In Table 1, we demonstrated that percent of halos (gelatin digestion points) was decreased gradually from 60±2.04 (negative control) to 44±1.66% (800 μM mercury) ($p<0.01$). The value of correlation between percentage of halos and LPO rate was -0.893 ($p<0.001$).

DISCUSSION

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 mercuric chloride have been a matter of concern because of potential wide human exposure consequent to its wide spread use (World Health Organization, 1989). However, at present, little is known of the exact anti-fertility mechanism of mercury effect on the sperm function in the human model.

It was established that mercury induced 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 conformation (Delnomdedieu *et al*., 1992). The results obtained from present study also indicate that mercury acted as a potential oxidant when to human sperm samples in a concentration-dependent manner. Metal ions as transition metals cause cellular damages via formation of OH, which is derived from O_2^- and hydrogen peroxide under Haber-Weiss reaction (Halliwel and Gutteridge, 1984).

The thiobarbituric acid-reacted MDA as an index of LPO damages 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 leads 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 induced a significant reduction in the epididymal sperm viability and motility.

We showed earlier that mercury supplementation to carp gill cell suspensions led to a significant decline in the GSH content, with an inverse proportion to oxidative condition and that protection could be provided by addition of antioxidants indicating a challenge between antioxidants and ROS-mediated LPO process (Arabi, 2004a). It may be assumed that mercury is able to provide free radicals as a powerful fuel for peroxidation cascade resulting in the depletion of cellular antioxidants like GSH and propagation of LPO process (De Flora *et al*., 1994). However, it needs further assessments.

Following peroxidation to plasma membrane, the ion exchanges may impaired, which is for maintenance of sperm movement (Rao *et al*., 1989). Oxidative stress-mediated damage to the sperm membrane may account for defective sperm function observed in a high proportion of infertility cases (Sharma and Agarwal, 1996). 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. Disruption in ATP supply through disorganization of mitochondrial membranes is a major cause of lowered sperm viability in the metal ion-treated sperm samples (Au *et al*., 2000). It may be concluded that ROS modify lipid structure in the sperm membranes resulting in altered viability and movement variables. A strong negative correlation was also seen between elevated LPO values and viability results ($r = -0.941$).

Oxidative stress does not simply disrupt the fertilizing capacity of spermatozoa, it attacks the integrity of sperm chromatin and to cause high frequencies of single and double-stranded DNA breaks (Aitken and Krause, 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 effects and intactness of sperm membrane and DNA integrity.

In the present study, a positive correlation was found between increased DNA breaks and LPO rate values indicating that ROS-mediated LPO in the semen samples simultaneously causing DNA breaks in the sperm chromatin. The use of acridine orange stain helped us to distinguish single-stranded from double-stranded DNA breaks in sperm nucleus. Almost all the DNA breaks (88%) were of double-stranded. 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 suspension (Arabi, 2004b). 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 modification 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 leukocyte (Grover *et al*., 2001). Present findings are consistent with the above observations.

A normal acrosome exocytosis in spermatozoa is essential for zona penetration and fertilization. Present 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, negative alterations in the sperm plasma membrane

due to peroxidation condition may be accounted for the absence of sufficient acrosome reaction in the mercury-treated sperm cells. It has been demonstrated that the inhibition of an outward-directed Ca^{2+}-ATPase in the head and flagellar membranes of mammalian spermatozoa would facilitate the Ca^{2+} accumulation in the spermatozoa, which is required for acrosome reaction and motility while spermatozoa are in the female's genital tract (Garcia *et al*., 1991; Li and Chen, 1996). This unique phenomenon may indicate that, spermatozoa during our study underwent a spontaneous release of their acrosomal enzymes prior to any contact with gelatin slides. As Ca^{2+}-ATPase is a membrane-bound enzyme so that any alteration in membrane structure by any oxidative agent such as metal ions may translate into inactivation of enzyme. However, it is reasonable to suggest that mercury may impose a severe lack of fertility potential to the human spermatozoa via impaired cell membrane and an abnormal pattern of acrosome enzymatic exocytosis.

The conclusion drawn from this *in vitro* study is that mercury proved to be a reproductive toxicant that induced peroxidation to the sperm membrane elements, declined viability, elevated DNA breaks and decreased percentage of acrosome reactions in men. It can be also concluded that the excessive amounts of mercury in the seminal plasma appears to be related to abnormal spermatozoal function. However, further research efforts need to be conducted to determine other aspects of mercuric infertility in human spermatozoa.

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