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Decreased *in vitro* fertility in male rats exposed to fluoride-induced oxidative stress damage and mitochondrial transmembrane potential loss

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ABSTRACT

Fluorosis, caused by drinking water contamination with inorganic fluoride, is a public health problem in many areas around the world. The aim of the study was to evaluate the effect of environmentally relevant doses of fluoride on *in vitro* fertilization (IVF) capacity of spermatozoa, and its relationship to spermatozoa mitochondrial transmembrane potential ($\Delta\Psi_m$). Male Wistar rats were administered at 5 mg fluoride/kg body mass/24 h, or deionized water orally for 8 weeks. We evaluated several spermatozoa parameters in treated and untreated rats: i) standard quality analysis, ii) superoxide dismutase (SOD) activity, iii) the generation of superoxide anion ($O_2^{\cdot -}$), iv) lipid peroxidation concentration, v) ultrastructural analyses of spermatozoa using transmission electron microscopy, vi) $\Delta\Psi_m$, vii) acrosome reaction, and viii) IVF capability. Spermatozoa from fluoride-treated rats exhibited a significant decrease in SOD activity (~33%), accompanied with a significant increase in the generation of $O_2^{\cdot}$ (~40%), a significant decrease in $\Delta\Psi_m$ (~33%), and a significant increase in lipid peroxidation concentration (~50%), relative to spermatozoa from the control group. Consistent with this finding, spermatozoa from fluoride-treated rats exhibited altered plasmatic membrane. In addition, the percentage of fluoride-treated spermatozoa capable of undergoing the acrosome reaction was decreased relative to control spermatozoa (34 *vs.* 55%), while the percentage fluoride-treated spermatozoa capable of oocyte fertilization was also significantly lower than the control group (13 *vs.* 71%). These observations suggest that subchronic exposure to fluoride causes oxidative stress damage and loss of mitochondrial transmembrane potential, resulting in reduced fertility.

Introduction

Fluoride is a naturally occurring component of ground water, and waters with naturally high levels of fluoride are found mostly at the base of high mountains, and in areas where the sea has made geological deposits (NRC, 2006). However, other sources, such as dental products, foods, and pesticides constitute a greater source of fluoride-exposure for most people (NRC, 2006). High groundwater fluoride concentrations have been reported in India, China, Spain, and Mexico, where levels are higher than 1.5 ppm (Del Razo et al., 1993; Gupta et al., 1993; Hardisson et al., 2001; Wang et al., 2007). Fluoride is rapidly absorbed from the gastrointestinal tract; with a half-life of about 30 min, calcified tissues such as bone and teeth are the main target of the toxic action due to preferred fluoride accumulation (NRC, 2006). However, in chronic fluoride-exposure the toxic effects in soft tissues have also been recognized. Fluoride-induced toxicity, including reproductive defects, has been reported by many groups. For example, Freni (1994) showed an inverse correlation between human fertility and fluoride levels in drinking water. Epidemiological data have also indicated that fluoride may adversely affect the reproductive systems of men living in endemic areas of fluorosis (Ortiz-Perez et al., 2003).

A variety of mechanisms have been proposed to explain fluoride-induced toxicity, including oxidative stress. Oxidative stress has been observed in soft tissues such as the liver, kidney, brain, and testes in animals (Ghosh et al., 2002; Guo et al., 2003; Shanthakumari et al., 2004; Krechniak and Inkielewicz, 2005; Mittal and Flora, 2007) and in people living in areas of endemic fluorosis (Shivarajashankara et al., 2001). Fluoride is thought to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase. Moreover, fluoride can alter glutathione levels (Chlubek, 2003; Lawson and Yu, 2003), often resulting in excessive production of reactive oxygen species (ROS) at the mitochondrial level, leading to damage of cellular components. Importantly, spermatozoa fertility depends on maintenance of mitochondrial transmembrane potential ($\Delta\Psi_m$) by the electron-transport chain (Wang et al., 2003), suggesting a mechanism for fluoride impact on fertility.

Oxidative stress has been reported to be associated with reproductive impairment (Aitken et al., 1993, 1998), and high levels of ROS lead to ATP depletion, motility loss, lipid peroxidation, and decreased fertility in spermatozoa (Aitken et al., 1992; de Lamirande et al., 1997).

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Spermatozoa are particularly susceptible to oxidative stress given by the high membrane content of unsaturated fatty acids as well as limited stores of antioxidant enzymes (Sanocka and Kurpisz, 2004; Baker and Aitken, 2005). Moreover, oxidative damage can seriously compromise the ability of spermatozoa to engage in sperm–oocyte fusion (Aitken et al., 1993).

Although several studies have shown the adverse effects of fluoride levels upon male reproduction (Chinoy and Narayana, 1994; Chinoy et al., 1995), the direct effects of fluoride on spermatozoa acrosome reaction and *in vitro* fertilization (IVF) have not been quantified. In addition, is not known if fluoride-exposure promotes the generation of ROS in spermatozoa. Here, we investigated the effect of fluoride-exposure on the reproductive ability of male rats and the relationship of oxidative damage and spermatozoa mitochondrial function.

Materials and methods

Chemicals

Bovine serum albumin fraction V (BSA), butylated hydroxytoluene (BHT), deferroxamine (DFA), dimethyl sulfoxide (DMSO), sodium fluoride (NaF), formaldehyde, hyaluronidase, hoechst 33342, human chorionic gonadotropin (hCG), lactic acid, sodium pyruvate, and thiobarbituric acid (TBA), were from Sigma Chemical Co. (St. Louis, MO). Pregnant mare serum gonadotropin (PMSG) (Folligon) was from Intervet, International B.V. (Boxmeer, Holland). Trichloroacetic acid (TCA) was from J.T. Baker (Phillipsburg, NJ). Coomassie blue G-250 from Fisher Scientific (Fair Lawn, NJ). RANSOD kit from Randox Laboratories, Ltd (Crumlin, UK). Dihydroethidium (DHE), sytox green and JC-1 kit were purchased from Molecular Probes, Invitrogen (Mount Waverley, Australia), and protein assay kit from Bio-Rad (Hercules, CA). All other chemicals used were from the highest purity commercially available.

Animals and experimental design

Male Wistar rats (75–99 g) and immature (5 weeks old) female Wistar rats were obtained from Harlan (Mexico, D.F.). Animals were maintained according to the Institutional (Cinvestav-IPN) Animal Care and Use Committee (CICUAL) in compliance with Guidelines for Use and Care of Laboratory Animals. Animals were maintained in groups of six per cage, on a 12 h/12 h light/dark cycle at constant temperature (22±2 °C) and humidity (50%), with food (LabDiet® 5013, PMI Nutrition International, St. Louis, MO) and water freely available in their home cages.

Male rats were administered subchronically with NaF at a dose equivalent to 5 mg fluoride/kg body mass/24 h by gavage or deionized water for the control group. The duration of fluoride-exposure was 8 weeks, since one spermatogenic cycle in the rat is 50±2 days; this way we are assured of the fluoride-exposure during at least a complete period of spermatogenesis in the rat.

Fluoride serum concentration

Fluoride serum concentration was examined at 1, 2, 4, 6 and 8 weeks of fluoride-exposure, by collection of 0.5 ml tail vein blood from each rat. Fluoride levels were measured with a potentiometric method using the ion selective electrode (Orion 9609) (Del Razo et al., 1993).

Spermatozoa isolation and capacitation

After 8 weeks of treatment, rats were euthanized by cervical dislocation, testes–epididymis–vas deferent complexes were dissected, and spermatozoa were isolated by flushing vas deferens and cauda epididymis lumens with 1 ml of phosphate buffered saline (PBS pH 7.4). Spermatozoa counts were determined using a Neubauer chamber. To induce capacitation, 10×10^6 spermatozoa/ml Enriched Krebs–Ringer Bicarbonate (EKRB) supplemented with 3 mg/ml BSA were incubated for 4 h at 37 °C in a high humidity incubator under 5% CO_2 (Bendahmane et al., 2002).

Spermatozoa quality

Sperm parameters including concentration, viability and progressive motility, were evaluated according to WHO guidelines (2001). Spermatozoa motility (percent of motile cells) was assessed by microscopic examination of ten random fields. Spermatozoa viability was determined by trypan blue exclusion assay. Spermatozoa concentrations were determined using a hemocytometer. Two aliquots (100–200 cells each) were separately counted for each animal.

SOD activity in spermatozoa

SOD was extracted from 10×10^6 spermatozoa, treated 1:1 with 0.1% Triton X100-PBS, incubated at 4 °C for 15 min. Samples were then centrifuged at 600 ×g for 8 min at 4 °C, and supernatants removed for measurement of SOD using the RANSOD Assay kit. This method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was measured by the degree of inhibition of xanthine to water and molecular oxygen at 505 nm using a spectrophotometer (VitaLab ECLIPSE Merck, Darmstadt, Germany). SOD activity was calculated using a standard curve, according to the manufacturer's instructions. The unit of activity of the assay was defined as the amount of SOD that inhibited the rate of formazan dye formation by 50%. The results presented as units per milligram of protein.

Measurement of $O_2^{\cdot-}$ generation

Levels of $O_2^{\cdot-}$ levels were measured by flow cytometry using DHE and SYTOX Green, as previously described (De Iuliis et al., 2006). DHE is a poorly fluorescent product of the two-electron reduction ethidium that, on oxidation, produces DNA-sensitive fluorochromes that generate a red nuclear fluorescence when excited at a wavelength of 510 nm. For the assay, 2×10^6 spermatozoa in EKRB medium with BSA were incubated with 3 μM DHE and 0.25 μM SYTOX Green in the dark at 37 °C for 1 h. Fluorescence was then measured for 10,000 cells using a flow cytometer (FACSCalibur system, Becton Dickinson; Franklin Lakes, NJ).

TBARS concentration in spermatozoa

Malondialdehyde concentration was used as an index of lipid peroxidation and was determined using the thiobarbituric acid reactive substances (TBARS) method (Buege and Aust, 1978). Briefly, 1 ml of 0.5% TBA, 5 μl of 3.75% BHT in methanol, and 5 μl of 1.5 mM DFA were added to 1 ml of spermatozoa suspension (2×10^6 cells). Samples were then heated in a boiling water bath for 20 min, cooled, and the absorbance was measured at 532 nm using a spectrophotometer (UV-Vis Lambda-2S Perkin-Elmer). Measurements are expressed as nmol TBARS/2×10^6 spermatozoa.

Transmission electron microscopy

Spermatozoa samples from control and fluoride-treated rats were fixed with 3% (v/v) glutaraldehyde in PBS buffer for 1 h at room temperature. Samples were then postfixed in 1% (v/v) osmium tetroxide in PBS for 1 h. The cells were rinsed in PBS, dehydrated through a grade ethanol series, and embedded in Spurr's resin. Resin blocks were thin-sectioned and double-stained with uranyl acetate and lead nitrate. The samples were examined using a JEM-1200 EXII transmission electron microscope at 60 keV (Jeol LTD; Tokyo, Japan). From each sample, ten thin sections were separately analyzed.

Spermatozoa mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using the fluorophore JC-1, a lipophilic cation that differentially labels mitochondria on the basis of membrane potential, with high potential leading to emission at 590 nm (red) and low membrane potential 525–530 nm (green), when excited at 488 nm. Three microliters of JC-1 in DMSO was added to each sample of 2×10^6 cells/ml in EKRB medium, and incubated for 30 min at 37 °C in a high humidity incubator under 5% CO_2. Fluorescence intensities were measured for 10,000 cells using a flow cytometer (FACSCalibur system, Becton Dickinson; Franklin Lakes, NJ).

Acrosome reaction

The ability of spermatozoa to undergo acrosome reaction was evaluated following 0 and 2 h of incubation in EKRB medium at 37 °C in a high humidity incubator under 5% CO_2. Spermatozoa from control and fluoride-treated rats were fixed with 4% paraformaldehyde in PBS for 10–15 min at room temperature. Spermatozoa were then centrifuged at 500 ×g for 5 min, and then washed twice with PBS. Aliquots were spread on microscope slides and air-dried at room temperature. Samples were then incubated in fresh Coomassie stain (0.22% Coomassie Blue, 50% methanol, 10% glacial acetic acid) for 5 min at room temperature. Afterwards slides were then washed with double-distilled water to remove excess dye. Slides were then air-dried, added with a drop glycerol:PBS (1:1), covered with a cover slip and sealed with nail polish. Cells were examined by phase-contrast microscopy at 100× magnification.

In vitro fertilization (IVF)

Egg recovery. Five weeks old female Wistar rats were superovulated by intraperitoneal injection of 20 IU PMSG, and 20 IU hCG 48 h later. Animals were euthanized 14–16 h after hCG injection by cervical dislocation. Uterine ovary–salpinge–horn complexes were dissected, ampullae punctured, and cumulus–eggs complex were extruded and placed in 0.1% (w/v) hyaluronidase/EKRB medium to remove cumulus cells. Cumulus-free eggs were pooled and washed with EKRB medium, and then incubated at 37 °C under 5% CO_2 until use. Approximately 40 eggs were obtained from each female. Only eggs with polar bodies and with intact zonae pelucidae were used for fertilization assays.

IVF assay. To assess spermatozoa fertility, fourteen eggs in 200 μl EKRB medium were inseminated with 10 μl of capacitated spermatozoa (1×10^5 cells) for controls or fluoride-treated groups, and were incubated for 4 h at 37 °C in a high humidity incubator under 5% CO_2. Samples were then fixed in 3% formaldehyde in PBS and stained with

Table 1
Weekly fluoride serum levels

Time (weeks)	Serum fluoride concentration (ppm)
0	<0.02
1	0.043±0.012
2	0.141±0.024
4	0.204±0.033
8	0.263±0.024

Data represent the means±SD of 6 animals per group.

20 μM Hoechst 33342 for 20 min. Samples were then washed three times in PBS, and then examined by fluorescence microscopy to assess fertilization. Eggs were considered fertilized when decondensed spermatozoa heads were detected within the egg cytoplasm.

Statistical analysis

Results are expressed as means±standard deviation (SD). The statistical significance of differences between the experimental groups was calculated by two-tailed *t*-test; *p* values<0.05 were considered significant. All analyses were performed using the statistical software Stata 8.0 (Stata Corporation).

Results

Water and food intake in the fluoride-exposed group were similar to the control group during exposure time. In consequence, no significant differences were observed in the body weight of rats exposed (data not shown).

Serum fluoride concentrations were measured in fluoride-treated male rats. Mean concentrations are presented in Table 1. Steady state fluoride serum levels were reached after 4 weeks of daily exposure to fluoride.

Fluoride-exposure affects the spermatozoa motility

Next we analyzed the overall quality of spermatozoa according to several parameters, summarized in Table 2. Only motility was significantly reduced in spermatozoa from fluoride-treated rats, compared with the control group (81±6.8 *vs.* 92±5.6, $p=0.027$). While spermatozoa viability and concentration exhibited a slight decrease following fluoride treatment, the change was not statistically significant.

Subchronic exposure to fluoride causes oxidative stress and damage in rat spermatozoa

Oxidative stress results when cellular antioxidant defenses become overwhelmed by ROS. To evaluate oxidative stress, the functional activity of SOD and generation of $O_2^{\cdot -}$ were assessed. As shown in Fig. 1A, (5 mg/kg/24 h, for 8 weeks) fluoride-exposure led to a significant decrease in total SOD activity, which was 3.28-fold lower than in the control group ($p=0.001$). In spermatozoa from rats exposed to fluoride, DHE fluorescence relative was 1.4-fold greater than in the control group ($p=0.001$), indicating increased $O_2^{\cdot -}$ levels in spermatozoa from fluoride-treated rats (Fig. 1B). We also examined lipid peroxidation, a marker of oxidative damage (Fig. 1C). Lipid peroxidation levels were increased by 1.5-fold in spermatozoa from fluoride-treated rats compared with controls ($p=0.001$). These observations suggest that fluoride-exposure causes oxidative stress and damage in rat spermatozoa.

Table 2
Assessment of spermatozoa parameters in rat exposed to fluoride

Sperm parameters	Control ($n=6$)	Fluoride ($n=6$)
Concentration (10^6 cells/ml)	41.1±14.2	30.6±13.5
Motility (%)	92±5.6	81±6.8*
Viability (%)	97±0.95	95±1.5

Values are mean±SD, *$p<0.05$ *vs.* control group.

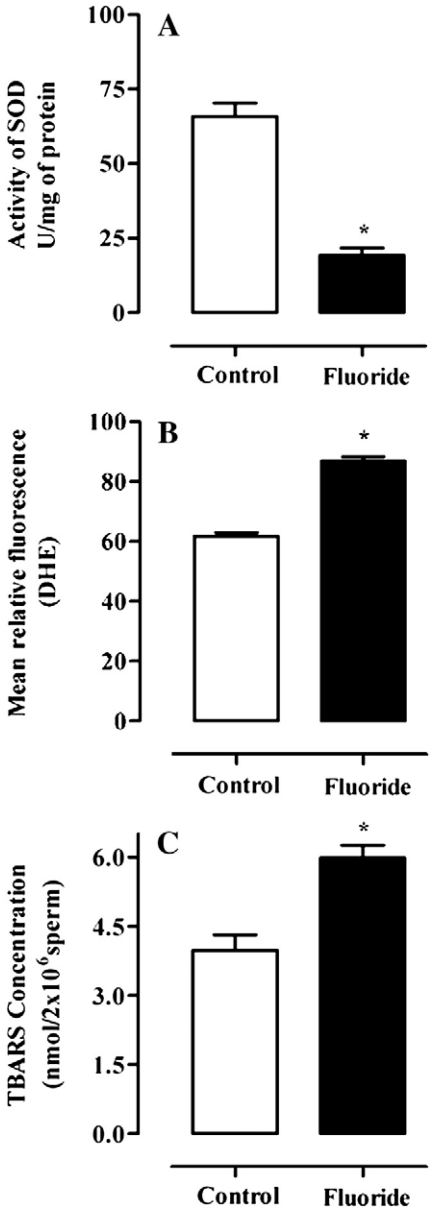

Fig. 1. Subchronic fluoride-exposure induces oxidative stress and oxidative stress damage in spermatozoa. A) Effect after 8 weeks of fluoride-exposure on SOD activity, measured by RANSOD assay. B) Effect of fluoride-exposure on $O_2^{\cdot}$ generation. $O_2^{\cdot}$ levels were measured by DHE/SYTOX green staining and flow cytometry. C) Effect of fluoride-exposure on lipid peroxidation, determined using the TBARS method. Data represent the means±SD of 6 animals per group, *$p<0.05$ *vs.* the control group.

Subchronic exposure to fluoride causes damage in the membrane of spermatozoa

Ultrastructural evaluations of spermatozoa from fluoride-treated and control rats were performed via transmission electron microscopy, and representative images are shown in Fig. 2. The control spermatozoa exhibited an intact plasmatic membrane along the cell (Fig. 2A). This normal appearance was visibly altered in spermatozoa from fluoride-treated rats. The cells showed a discontinuous plasmatic membrane along the sperm head (Fig. 2B).

Fluoride-exposure affects mitochondrial function in spermatozoa

Loss of $\Delta\Psi_m$ is a sensitive indicator of mitochondrial damage. We evaluated changes in spermatozoa mitochondria following fluoride-

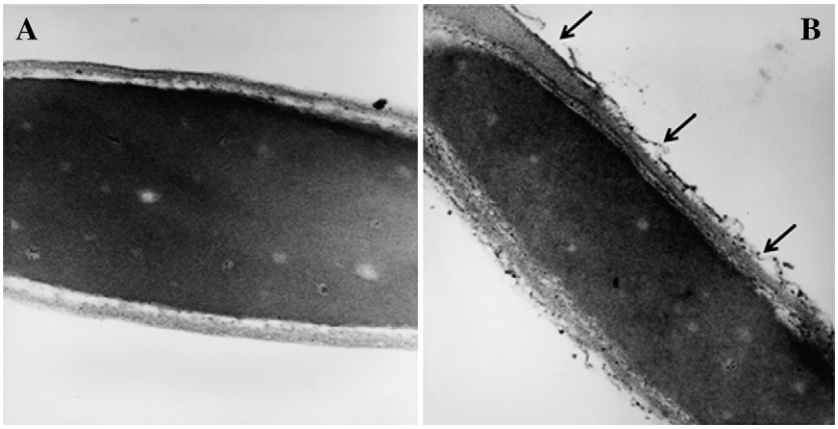

Fig. 2. Effect of subchronic fluoride-exposure on ultrastucture of spermatozoa. Spermatozoa isolated from control (A) or fluoride-treated rats (B) during 8 weeks were examined by transmission electron microscopy (×20,000). Plasma membranes of control spermatozoa were intact. While the membranes showed visible alterations in fluoride-treated spermatozoa (arrows).

exposure. $\Delta\Psi_m$ was measured using the fluorophore JC-1, which exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red. We observed a 33% decrease in $\Delta\Psi_m$ in spermatozoa from fluoride-treated rats compared with the control group (Fig. 3). These results indicate that fluoride-exposure can cause a decrease in spermatozoa mitochondrial function.

Fluoride-exposure leads to reduced ability to undergo the acrosome reaction and IVF

Lipid peroxidation affects spermatozoa membrane fluidity and flexibility, since membrane structure plays a pivotal role in fertilization. We evaluated the ability of treated and untreated spermatozoa to undergo the acrosome reaction and IVF. The percentage of spermatozoa undergoing the acrosome reaction was evaluated at 0 and 2 h of capacitation. At 2 h of capacitation, the percent of spermatozoa having undergone the acrosome reaction was decreased by 1.6-fold in the fluoride-exposed rats, compared with the control group ($p=0.004$) (Fig. 4). Next, we examined the ability of treated and untreated spermatozoa to fertilize zona-intact eggs by IVF. Spermatozoa from rats exposed to fluoride exhibited a significantly lower ability to fertilize eggs compared to the control group (13±5.10% *vs.* 72±4.69%), (Figs. 5 and 6).

Discussion

The aim of this study was to evaluate whether the oxidative stress caused by fluoride-exposure is correlated with reduced fertility. Fluoride-induced reproductive defects have been reported previously for experimental models and humans (Al-Hiyasat et al., 2000; Verma and Guna Sherlin, 2002; Ortiz-Perez et al., 2003). However, these effects were observed in heavily fluoride-exposed experimental scenarios (>10 mg/kg/day). In the present study, we used an environmentally relevant dose of fluoride, since serum concentrations were comparable to levels found in environmentally-exposed populations, which are in the range of 0.01 to 1.2 ppm (Bhagaban and Raghu, 2005).

Spermatozoa quality is a major factor in successful IVF. Here, we verified that spermatozoa motility was significantly reduced as a result of fluoride-exposure. Although conventional semen analysis provides considerable information, new methods are needed to more easily and reliably predict spermatozoa fertilization capacity. Spermatozoa motility has been proposed to be associated with the functional status of spermatozoa mitochondria (Gravance et al., 2001). Indeed,

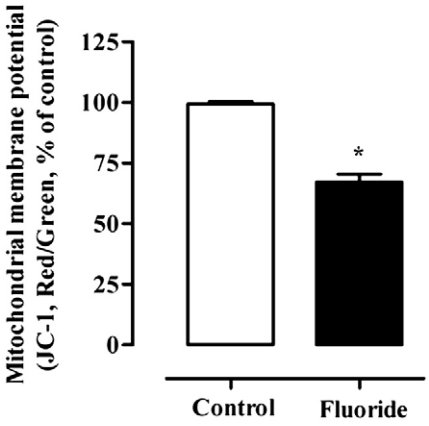

Fig. 3. Subchronic fluoride-exposure leads to decreased membrane potential ($\Delta\Psi_m$). Graphical representation of $\Delta\Psi_m$ for treated spermatozoa relative to control spermatozoa. $\Delta\Psi_m$ is defined as the ratio of flow cytometric fluorescence intensities for red (FL2) and green (FL1) channels for JC-1-stained sperm samples. $\Delta\Psi_m$ was evaluated in spermatozoa from rats exposed an unexposed to fluoride after 8 weeks. Results represent three independent experiments performed by triplicate. Data represent the means±SD of 6 animals per group,*$p<0.05$ *vs.* the control group.

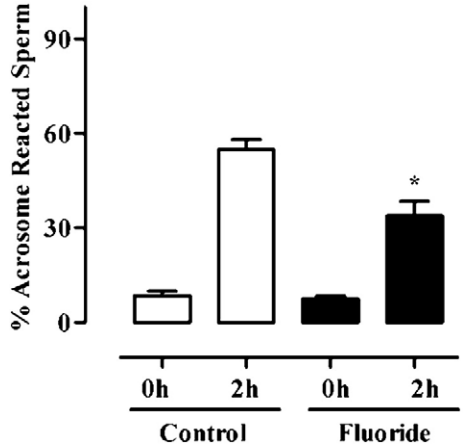

Fig. 4. Effect of subchronic fluoride-exposure on acrosome reaction. Acrosome reaction was evaluated in spermatozoa from rats exposed an unexposed to fluoride after 8 weeks. Percent of spermatozoa undergoing the acrosome reaction spontaneously in capacitation conditions. The acrosome reaction was evaluated at 0 and 2 h of incubation in the EKRB supplemented with BSA 3 mg/ml as described in materials and methods. At least six rats for each group were examined in duplicate. *$p<0.05$ *vs.* the control group.

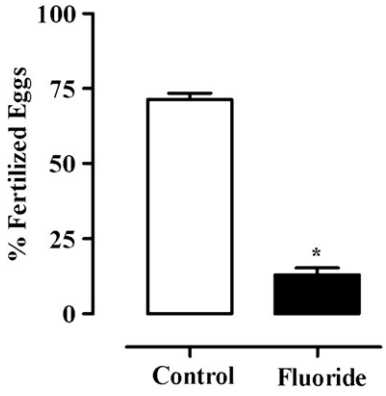

Fig. 5. Effect of subchronic fluoride-exposure on fertilization. Spermatozoa isolated from treated rats to fluoride during 8 weeks were tested for their ability to fertilize zona-intact eggs *in vitro*. A total of 120 eggs were examined for each experiment group, over the course of three independent experiments, $*p<0.05$ *vs.* the control group.

mitochondrial alterations can result in decreased spermatozoa motility since motility is ATP-dependent (Ford and Harrison, 1981; Ford and Rees, 1990). Interestingly, studies have suggested that mitochondria are the major target of fluoride ion toxicity in the human kidney (Cittanova et al., 1996), and in rat liver and pancreas (Dabrowska et al., 2004). We also evaluated spermatozoa $\Delta\Psi_m$, which is widely used to characterize the functional status of mitochondria (Gravance et al., 2001), and found a significant decrease in $\Delta\Psi_m$ in fluoride-treated spermatozoa, indicating mitochondrial dysfunction. Mitochondrial dysfunction can promote high ROS production, and an inverse correlation between $\Delta\Psi_m$ and ROS levels in spermatozoa in semen samples from patients with abnormal semen parameters has been observed (Wang et al., 2003).

Advances in the understanding of male infertility have implicated oxidative stress as a major causative factor (Aitken et al., 1993, 1998). Changes in the cellular redox status toward oxidative conditions may occur as a result of overproduction of ROS or a deficiency in antioxidant system. SOD acts as an important line of antioxidant defense by catalyzing the dismutation of $O_2^{\cdot-}$ into oxygen and hydrogen peroxide. In this study, subchronic exposure to fluoride in rats caused a significant decrease in total SOD activity in spermatozoa. This is the first evidence of fluoride-decreased SOD in spermatozoa. This may be due to fluoride acting as an inhibitor of Mn-SOD and Cu/Zn SOD, their proposed mechanism involves its binding to the divalent cofactors in active site on SOD (Wilde and Yu, 1998; Lawson and Yu, 2003), although also a decrease in SOD mRNA has been observed in pigs exposed to 250 mg/kg of fluoride (Zhan et al., 2006). Inactivation of SOD would lead to increased levels of $O_2^{\cdot-}$ within the mitochondria which, could lead to oxidation of key mitochondrial proteins and ultimately mitochondrial dysfunction and cell death.

In addition to evidence of fluoride-induced oxidative stress in spermatozoa, we observed a significant increase in TBARS concentration of spermatozoa from rats exposed to fluoride, suggesting oxidative damage had occurred. Here, oxidative damage to spermatozoa plasma membrane was evidenced by electron microscopy. Consistent with this observation, a positive correlation has been observed between the concentrations of fluoride and TBARS in several tissues, including the testes (Krechniak and Inkielewicz, 2005). Recently, lipid peroxidation has been proposed to be an important contributor to mitochondrial dysfunction rather than the inhibition of the mitochondrial electron transport (Sen et al., 2006). There is evidence that oxidative damage and mitochondrial dysfunction in spermatozoa play an important role in fluorosis.

During fertilization, only capacitated spermatozoa can undergo the acrosome reaction after binding to the egg zona pellucida, a process that enables spermatozoa to penetrate and fertilize the egg (Primakoff and Myles, 2002). Capacitation process of mammalian spermatozoa involves a series of changes, including reorganization of membrane proteins, metabolism of membrane phospholipids, reduction of membrane cholesterol levels, tyrosine phosphorylation, and generation of low and controlled levels of $O_2^{\cdot-}$ (Yanagimachi, 1994; de Lamirande and Gagnon, 1995). Addition of exogenous NADPH to spermatozoa suspensions results in increase in the level of tyrosine phosphorylation, suggesting that capacitation is a redox-regulated event (Aitken et al., 1995). However high levels of ROS can have deleterious effects on spermatozoa (de Lamirande and Gagnon, 1995), and are associated with reduced

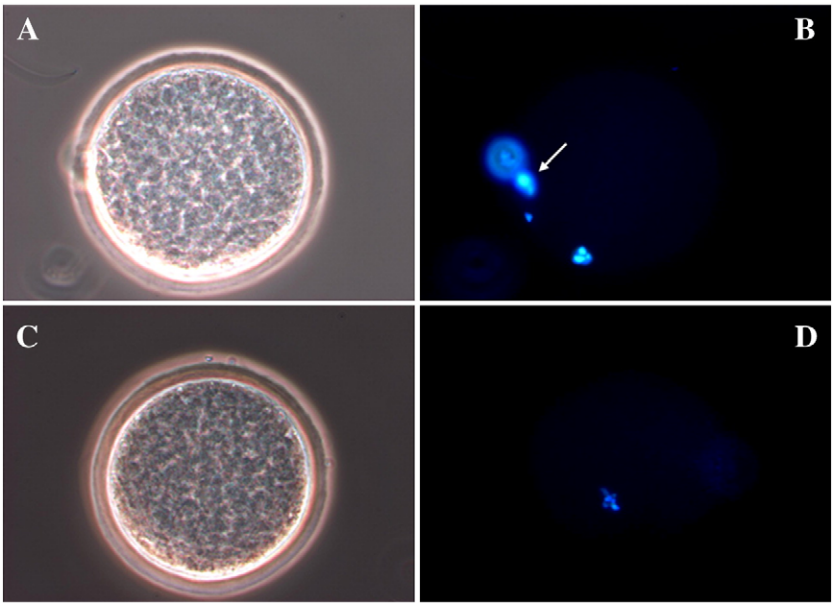

Fig. 6. Effect of subchronic fluoride on IVF capability. Representative phase-contrast (A, C) and fluorescence (B, D) micrographics of IVF embryos (×4000). Zona-intact eggs were incubated for 4 h with spermatozoa from control or fluoride-exposed rats and stained with Hoechst 33342. (A, B) Eggs fertilized with control spermatozoa. A swollen spermatozoa nucleus was observed in the egg cytoplasm (arrow). (C, D) Eggs fertilized with spermatozoa exposed to fluoride during 8 weeks. No evidence of swollen spermatozoa nuclei was observed in egg cytoplasms.

motility and fertilization potential (Aitken et al., 1998). We found that fluoride caused a significant increase in the generation of $O_2^{\cdot -}$ in spermatozoa. This is the first evidence of fluoride-induced generation of $O_2^{\cdot -}$ in spermatozoa. Consistent with this observation, another group reported that fluoride stimulates production of $O_2^{\cdot -}$ in neutrophils (Wolfl et al., 1996). These results, suggest that overproduction of ROS may be from mitochondria, since mitochondrial dysfunction promote the increase of ROS. In addition, the SOD activity may be decreased by the overproduction of ROS or/and, SOD inhibition by fluoride (Wilde and Yu, 1998; Lawson and Yu, 2003). Moreover, a NADPH-oxidase has emerged as a potential mediator of ROS production by spermatozoa (Geiszt and Leto, 2004). Participation of fluoride-exposure in ROS generation deserves major investigation.

Because a small amount of ROS has a fundamental role in activation of signal transduction pathways leading phosphorylation in tyrosine residues of proteins in the spermatozoa, an overproduction in ROS may alter this event. Consistent with this observation, fluoride treatment has recently been shown to decrease the percentage of spermatozoa undergoing head tyrosine phosphorylation and actin polymerization in the cortical acrosomal region during *in vitro* capacitation (Dvořákova-Hortová et al., 2008).

Here, we found that fluoride-exposure caused a decrease in spermatozoa fertilization capability. The decrease in fertility and the ability to undergo the acrosome reaction may result from oxidative damage to the plasma membrane, which would lead to a total loss of membrane fluidity and integrity, since both processes are dependent on membrane fusion. This would reduce the number of spermatozoa available to compete in the membrane fusion events associated with fertilization, thereby decreasing overall fertility.

In summary, our results indicate that fluoride-exposure can cause mitochondrial dysfunction, oxidative stress, and oxidative damage. These effects lead to reduced fertility of rat spermatozoa *in vitro*.

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