Methyl-parathion decreases sperm function and fertilization capacity after targeting spermatocytes and maturing spermatozoa

Piña-Guzmán B[a], Sánchez-Gutiérrez M[b], Marchetti F[c], Hernández-Ochoa I[d], Solís-Heredia MJ[a], and Quintanilla-Vega B[a]*.

[a] Sección Externa de Toxicología, CINVESTAV, Av. IPN #2508, Col. Zacatenco, México, D.F., 07360, México.
[b] Área Académica de Medicina, Instituto de Ciencias de La Salud, Universidad Autónoma del Estado de Hidalgo, Abasolo 600, Pachuca, Hidalgo, 42000, México.
[c] Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mailstop 977R250, Berkeley, CA, 94720
[d] Department of Veterinary Biosciences, University of Illinois, 3209 Veterinary Medicine Basic Sciences Building, 2001 S. Lincoln Ave. Urbana, IL 61802.

*Corresponding author: Betzabet Quintanilla-Vega, Sección Externa de Toxicología, CINVESTAV, Av. IPN #2508, Colonia Zacatenco, México D.F., 07360, México. Tel. (+52) 55 5747-3800 Ext. 5446. Fax. (+52) 55 5747-3395. *e-mail*: mquintan@cinvestav.mx

ABSTRACT

Paternal germline exposure to organophosphorous pesticides (OP) has been associated with reproductive failures and adverse effects in the offspring. Methyl parathion (Me-Pa), a worldwide-used OP, has reproductive adverse effects and is genotoxic to sperm. Oxidative damage has been involved in the genotoxic and reproductive effects of OP. The purpose of this study was to determine the effects of Me-Pa on spermatozoa function and ability to fertilize. Male mice were exposed to Me-Pa (20 mg/kg bw, i.p.) and spermatozoa from epididymis-vas deferens were collected at 7 or 28 days post-treatment (dpt) to assess the effects on maturing spermatozoa and spermatocytes, respectively. DNA damage was evaluated by nick translation (NT-positive cells) and SCSA (%DFI); lipoperoxidation (LPO) by malondialdehyde production; sperm function by spontaneous- and induced-acrosome reactions (AR); mitochondrial membrane potential (MMP) by using the JC-1 flurochrome; and, fertilization ability by an *in vitro* assay and *in vivo* mating. Results showed alterations in DNA integrity (%DFI and NT-positive cells) at 7 and 28 dpt, in addition to decreased sperm quality and a decrease in induced-AR; reduced MMP and LPO was observed only at 7 dpt. We found negative correlations between LPO and all sperm alterations. Altered sperm functional parameters were associated with reduced fertilization rates at both times, evaluated either *in vitro* or *in vivo*. These results show that Me-Pa exposure of maturing spermatozoa and spermatocytes affects many sperm functional parameters that result in a decreased fertilizing capacity. Oxidative stress seems to be a likely mechanism of the detrimental effects of Me-Pa in male germ cells.

Key words. methyl-parathion, sperm function, fertilization, oxidative stress.

Abbreviations. AR = Acrosome Reaction; dpt = days post-treatment; %DFI = percentage of spermatozoa with DNA Fragmentation Index; IVF = *in vitro* fertilization; FR = fertilization rate; LPO = lipoperoxidation; OP = organophosphorous pesticides; MMP = mitochondrial membrane potential; Me-Pa = Methyl parathion; NT = nick translation.

Introduction

Epidemiological studies in the last decades have shown that fertility is declining in many countries (Jensen et al., 2000) and there has been substantial interest in the potential adverse effects of exposure to environmental hazardous chemicals, including pesticides, on male reproduction (Carlsen et al., 1992; Irvine, 1994; Adami et al., 1996; De Mouzon et al., 1996). Organophosphorous (OP) compounds are a broad group of chemicals widely used in agriculture as pesticides, in medicine as antihelminthics, in the airline industry as additives to hydraulic fluid and jet engine oil, and as chemical warfare agents (Storm et al., 2000). Thus, the possible toxicity of human exposure to OP compounds has aroused great concern.

Mammalian toxicity of OP has been widely reviewed (Gallo and Lawryk, 1991). The acute toxic action of these compounds involves their metabolic conversion to the bioactive oxygen analogues, the oxons (Jokanovik, 2001), and inhibition of acetylcholinesterase (AChE) followed by accumulation of acetylcholine (Rubbin et al., 2002). More recently, it has been reported that OP produce oxidative stress in different tissues, such as liver, blood and brain (Akhgari et al., 2003; Sharma et al., 2005; Fortunato et al., 2006) through the formation of reactive oxygen species (ROS) (Banerjee et al., 1999; Ranjbar et al., 2002).

Despite the large amount of OP toxicological data, the effects of OP compounds on the mammalian reproductive system been reported in recent years (Soranno and Sultatos, 1992). The exposure to OP results in the alteration of the antioxidant systems in testes (Debnath and Mandal, 2000) and production of lipoperoxidation (LPO) in spermatozoa (Piña-Guzmán et al., 2006). Low levels of ROS produced by spermatozoa are needed for physiological reactions such as those controlling phosphorylating events associated with sperm capacitation and acrosome reaction (AR) (Aitken et al., 1998; Aitken, 1999). However, excessive production of ROS and oxidative stress has been associated with defective sperm function and male infertility (Sikka et al., 1995). Additional mechanisms, such as those related to the alkylating (Dedek et al., 1984; Mehl et al., 2000) and phosphorylating (Piña-Guzmán et al., 2005) properties of OP compounds, have also been involved in their genotoxicity and damage to sperm.

Methyl parathion (Me-Pa; *o,o* dimethyl *o*-4-nitrophenyl phosphorothioate) is an OP classified as extremely toxic by WHO, and is being used in many countries to spray cotton, paddy fields, vegetables and fruits. Concern for its adverse effects on human reproduction has increased because of its genotoxic effect in somatic (Rupa et al., 1990) and sperm cells (Mathew et al., 1992; Piña-Guzmán et al., 2006). Studies in men and animals have shown that Me-Pa alone, or in combination with other OP, alters male reproductive function, particularly semen quality, sperm chromatin condensation, and hormonal balance (Padungtod et al., 2000; Sánchez-Peña et al., 2004; Narayana et al., 2005; Piña-Guzmán et al., 2006). Several studies have reported the genotoxic potential of Me-Pa on sperm cells (Mathew et al, 1992; Narayana et al., 2005; Piña-Guzmán et al., 2006); however, the direct impact of *in vivo* exposure to Me-Pa on susceptible stages of spermatogenesis and the consequences on functional parameters and fertilizing ability of spermatozoa has not been determined yet. In the current study, we investigated the effects of male exposure to Me-Pa on the function and fertilizing capacity of male germ cells and their relationship with oxidative damage.

Materials and Methods

Chemicals. Chemical grade (98% purity) Me-Pa was obtained from Chem. Service (West Chester, PA). 5,5′-dithiobis(2-nitro-benzoic acid) (TNB), thiocholine iodide, quinidine sulfate salt dehydrate, dithiothreitol (DTT), DNase I, colcemid, γ-aminobutyric acid (GABA), sodium pyruvate, lactic acid, bovine serum albumin fraction V (BSA), human chorionic gonadotropin (hCG), bovine testicular hyaluronidase, colchicine (CAS No. 64-68-8), and Hoechst 33342 were purchased from Sigma Chem. Co. (St Louis, MO), and acridine orange (AO) and SARH-FITC were from Amersham (Amersham, UK). Lipid Peroxidation Assay Kit was from Calbiochem (La Jolla, CA). DNA polymerase I and dUTP's, including biotin-16-dUTP, were purchased from Roche Applied Science (Mannheim, Germany). Equine chorionic gonadotropin (eCG) (Folligon) was from Intervet (Mexico, City). Formaldehyde was from J.T. Baker (Xalostoc, Mexico). Coomassie Blue G-250 was purchased from Bio-Rad (Richmond CA) and 4,6-iamidino-2-phenylindole (DAPI II) from VYSIS (Des Plaines, IL).

Animals. Male ICR-CD1 mice (10- to 12-weeks old) were obtained from CINVESTAV-IPN animal facilities. Mice were maintained in filtered cages under standard conditions of temperature (22 ± 2°C), relative humidity of 60 ± 10%, normal photoperiod (12-h dark/12-h light), and had free access to food and water. All animal procedures were approved by CINVESTAV-IPN Animal Care and Use Committee (CICUAL, for its initials in Spanish) in accordance with the guidelines of Mexican Official Norm on animal protection (NOM-062-ZOO-1999).

Experimental design. Mice were randomly divided into groups of six animals. Me-Pa was dissolved in corn oil and administered via intraperitoneal (i.p.) injection as a single dose of 20 mg/kg body weight (bw). This dose, which correspondes to 1/3 of the LD_{50} in mice(ref), was the highest used in our previous study where dose-dependent effects of single doses of Me-Pa on sperm quality and DNA damage were observed (Piña-Guzmán et al., 2006). Control animals received the vehicle only. Twelve exposed and twelve control mice were euthanized per time point (see below). Experimental analyses were conducted in at least two independent experiments.

Sperm isolation. Mice were euthanized at 7 or 28 days after Me-Pa exposure. This design allowed us to assess the effects on cells at different stages of maturation at the time of Me-Pa exposure: 7 days post-treatment (dpt) corresponded to late spermatids and maturing spermatozoa as the target cells, whereas 28 dpt corresponded to pachytene spermatocytes. Cauda epididymis-vas deferens were excised and freed of the fat pad, blood vessels and connective tissue and sperm cells were flushed with 1 ml of phosphate-buffered saline PBS (for sperm quality and DNA integrity; six control and six treated animals), or M16 medium at 37°C (for LPO, mitochondrial function, fertilization assays and acrosome reaction assays; six control and six treated animals).

Sperm quality evaluation. Sperm evaluation included cell concentration, viability, progressive motility and normal morphology according to WHO guidelines (1992).

Sperm chromatin structure assay (SCSA). Sperm DNA integrity was assessed by the Sperm Chromatin Structure Assay (SCSA) described by Evenson and Melamed (1983). SCSA measures the susceptibility of sperm DNA to *in situ* acid-induced denaturation by multiparameter flow cytometric analysis after staining with the DNA-specific fluorescent dye acridine orange (AO), which fluoresces green (515–530 nm) when intercalated into native double-stranded DNA, and emits a red fluorescence (>630 nm) when intercalated into denatured single-stranded DNA. The extent of DNA denaturation was quantified by the DNA Fragmentation Index (DFI), which is the ratio of red to red + green fluorescence, and the extent of DNA denaturation is expressed as the percentage of sperm with DFI (%DFI). Five thousand cells were analyzed per sample at a cell flow rate of less than 200 cells/s. Data were acquired 3 min after initiation of staining in list-mode and analyzed using the SCSASoft software (SCSA Diagnostics, Inc., Brookings, SD).

Nick Translation Assay. The nick-translating procedure was performed according to Sumner et al. (1990). Spermatozoa spread on slides were washed twice PBS, fixed in methanol/acetic acid (3:1 v/v) and air dried. Spermatozoa were then treated with 10 mM DTT in 10 mM Tris-HCL, pH 8.0, for 30 min, and *in situ*-nick translation was performed using the polymerase mixture (DNA polymerase I-endonuclease-free in 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 10 µM each of dATP, dCTP, dGTP and biotin-16-dUTP). A negative-control (omitting DNA polymerase I) and a positive-control (incubation with DNase I before adding the polymerase mixture) were included. Streptavidin (SAHR)-fluorescein isothiocyanate (FITC) was used to detect the incorporation of biotinylated d-UTP (NT-positive cells). After staining, 300 randomly selected cells were analyzed with an Olympus BX40 fluorescence microscope using 485 and 520 nm excitation and barrier filters, respectively.

Lipid peroxidation. Sperm suspensions at a concentration of 5×10^6 cells/ml were assessed for malondialdehyde (MDA) production as a measure of LPO, using the Lipid Peroxidation Assay Kit according to the manufacturer's instructions for cell lysates; volumes were modified for a 96-well microplate assay. This assay is capable of

detecting MDA which reacts with a chromogenic reagent (N-methyl-2-phenylindole) yielding a stable chromophore with maximal absorbance at 586 nm.

Acrosome reaction (AR). Percentage of spermatozoa without acrosome (acrosome-reacted) was measured using Coomassie Blue G-250 as described by Larson and Miller (1999). Briefly, sperm samples were incubated under capacitation conditions (M16 supplemented with 4 mg/ml BSA) for 0 or 60 min, to evaluate initial- or spontaneous-AR, respectively. To evaluate the induced-AR, spermatozoa were capacitated for 60 min and then treated with 0.5 μm GABA for 15 min. Fixation was made in 7.5% formaldehyde in PBS (pH 7.5) for 10 min followed by centrifugation at 9000 rpm for 1 min. The sperm pellet was washed and dissolved in ammonium acetate (100 mM, pH 9.0). After an additional centrifugation, the sperm pellet was resuspended in 20-50 μl of PBS, spread on glass slides, and air-dried. Slides were then stained with 0.22% (w/v) Coomassie Blue-G250, 50% methanol, 10% glacial acetic acid and 40% water for 2 min, gently rinsed with deionized water until they appeared blue, air dried, and then mounted with Cytoseal-60 mounting medium. To calculate the percentage of AR, at least 100 sperm cells per sample were scored at random and examined by phase-contrast microscopy at 100X magnification. Cells were classified as acrosome-intact (dark blue staining of the acrosomal vesicle) or acrosome-reacted (no staining in the acrosomal region).

Mitochondrial membrane potential (MMP). Sperm mitochondrial activity was evaluated by staining with the lipophilic cationic dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazoyl carbocyanine iodide). This fluorescent dye selectively enters into healthy mitochondria with high trans-membrane potential and accumulates there as JC-1 aggregates, which emit an intense red fluorescence. When the trans-membrane potential collapses in damaged mitochondria, the JC-1 dye can no longer accumulates and remains in the cytoplasm in a monomeric form that emits an intense green fluorescence. Sperm samples containing 1 x 10^6 cells/ml were incubated with 7 μl of 2 μM JC-1 for 15-20 min at 37°C in a high humidity incubator under 5% CO_2. After incubation, samples were immediately assessed for red and green fluorescence using a flow cytometer (FACSCalibur system, Becton Dickinson; Franklin Lakes, NJ). Forward

scatter (FS; approximate cell size) and side scatter (SS; cell complexity or granularity) of the sperm population were determined. A total of 10,000 gated events (based on FS and SS) were analyzed per sample; sample running rates were approximately 100–300 events per second. A 488 nm filter was used for excitation of JC-1; emission filters of 530 nm (green) and 590 nm (red) were used, respectively, to quantify the population of sperm with green and orange fluorescence. Frequency plots were prepared for FL1 (green) and FL2 (red) to determine the percent of the population stained green and red.

Egg collection for in vitro fertilization assay (IVF). Female ICR-CDI mice, 4-5 weeks old, were induced to superovulate by i.p. injection of 7.5 IU eCG (equine chorionic gonadotropin) followed by 5 IU hCG (human chorionic gonadotropin) 48 h later. Female mice were euthanized by cervical dislocation 16-18 h after hCG injection. Oviducts and attached ovaries were dissected and suspended in M16 medium supplemented with 4 mg/ml BSA. In each oviduct, the ampulla was punctured, and the oviductal cumulus masses were teased out and placed in 0.1% (w/v) hyaluronidase-M16 medium in order to remove cumulus cells. Then, cumulus-free eggs were washed with medium to remove hyaluronidase.

Egg insemination. Spermatozoa (25 x 10^6 cells/ml) were incubated in capacitating medium (M16 supplemented with 4 mg/ml BSA) until spontaneous-AR was observed (1 h at 37°C, 5% CO_2) and then were used for insemination. To assess fertilization, in a standard slide with one (or two) polished spherical depression(s) of approximately 0.5–0.8 mm depth, 20 eggs were resuspended in 300 μl M16 medium and inseminated with 10 μl of sperm suspension (2.5 × 10^5 cells) from control or treated mice. Gametes were co-incubated for 8 h (to evaluate the fertilization rate, FR) and 20 h (to evaluate the cell division rate) in a humidified incubator under light mineral oil at 37°C and an atmosphere of 5% CO_2–95% air. Sperm-egg complexes were gently rinsed with 100 μl M16 medium to remove loosely adherent spermatozoa and loaded onto a microscope slide. Samples were fixed 1:1 (v/v) in 3% formaldehyde for 12 h and used to assess the FR. Fixed samples were stained with 20 μM Hoechst 33342 for 20 min, washed three times in PBS, and then examined by fluorescence microscopy. According to the morphological

appearance, eggs were classified as fertilized when decondensed spermatozoa heads within the egg cytoplasm were observed or when they had two well defined pronuclei or were at the two-cell embryo stage showing two blastomeres of similar size. Cells exhibiting nuclear fragmentation and cellular debris enclosed by the zona pellucida were classified as degenerated oocytes or embryos (Tarin et al., 2002). Single cells without visible pronuclei and exhibiting normal morphology were categorized as unfertilized eggs. The FR was calculated as the number of pronuclear zygotes and two-cell embryos divided by the total number of eggs. Evaluations were made by phase-contrast microscopy.

In vivo fertilization assay. For this protocol, three independent experiments were done each using fifteen males. Males were tested for their mating ability 1 day before Me-Pa exposure (time 0) and then mated at 7 and 28 days after Me-Pa exposure. After the hCG injection to females, as described above, male and female mice were caged together (1:1) for 8 h. Mating pairs were then separated, and females were checked for the presence of vaginal plugs. Twenty four hours after hCG injection, mated females received an i.p. injection of 0.08 mg colchicine in 0.2 ml distilled water to arrest zygotic development at the first-cleavage metaphase stage, and they were euthanized by CO_2 inhalation 6 h later. Zygotes collected from each group of females were pooled and fixed according to the mass harvest procedure described by Mailhes and Yuan (1987) and classified into one of the following five groups according to their appearance (Marchetti and Wyrobek, 2003): unfertilized oocytes (oocytes with meiotic chromosomes or degenerating chromatin without a sperm head or tail); meiotic eggs (zygotes showing female meiotic chromosomes and a sperm head or tail); degenerated zygotes (zygotes with degenerating chromatin and a sperm head or tail, or fragmented pronuclei); pronuclei (zygotes with two well defined pronuclei showing the difference in size between paternal (larger) and maternal (smaller) pronuclei); and zygotes (zygotes with mitotic chromosomes). Evaluations were made by phase-contrast microscopy in three independent slides from each experimental group.

Statistical analyses. Results are expressed as mean ± SD. Data were analyzed by the Student t test. Correlations between Me-Pa effects on sperm chromatin structure, DNA, MMP, AR, and fertilizing ability and oxidative stress, as possible mechanism of action, were performed using bivariate analyses. All parameters were correlated by non-parametric Spearman's analysis. The STATA program version 8.0 (Stata Corp., College Station, TX) was used for all statistical calculations. Significance was set at $p < 0.05$.

Results

Me-Pa exposure impaired sperm quality

Sperm quality parameters were analyzed to provide a measure of the capacity of Me-Pa (20 mg/kg) exposure to exert a detrimental effect on male reproductive function. Control animals showed 86, 95 and 95% sperm motility, viability and normal morphology, respectively. As shown in Figure 1, at 7 dpt sperm motility and viability were decreased 23 and 15% ($p<0.05$), respectively. Significant decreases ($p<0.05$) in sperm motility (36.4%), viability (18.2%) and normal morphology (3%; predominantly head and mid piece abnormalities) were also observed at 28 days after Me-Pa treatment.

Sperm mitochondrial membrane potential is decreased in Me-Pa treated mice

We assessed mitochondria activity by means of JC-1fluorescence. Spermatozoa from Me-Pa-treated mice evaluated at 7 dpt exhibited ~29% lower MMP than spermatozoa from the control group, whereas spermatozoa collected at 28 dpt showed similar levels of mitochondrial activity than control cells (Figure 2).

Me-Pa reduces the induced-AR of spermatozoa

It is known that only acrosome-reacted spermatozoa can fertilize the oocyte (Yanagimachi, 1994); therefore, evaluating the percentage of spermatozoa capable of undergoing AR is a good indicator of their fertilizing ability. The percentage of spermatozoa showing spontaneous-AR was evaluated at 60 min after sperm capacitation and then spermatozoa were challenged with 0.5 μM GABA for 15 min to induce the AR in order to mimic natural conditions. We found that spermatozoa from

mice euthanized at 7 dpt showed similar values of spontaneous-AR than spermatozoa from the control group; however, an increase in the percentage of spermatozoa with spontaneous AR was observed at 28 dpt, compared to the control group (42.8% *vs* 32.5%) ($p<0.05$) (Figure 3). On the other hand, the percentages of spermatozoa capable of undergoing induced-AR in response to GABA decreased at both times, 7 dpt (3.3% *vs* 28%) and 28 dpt (2.5% vs 28.2%) compared to the corresponding control groups (Figure 3).

Sperm chromatin integrity was altered in Me-Pa treated mice

It has been documented that sperm DNA damage is inversely associated with IVF success (Sun et al., 1997); therefore, Me-Pa effects on mouse sperm chromatin structure were evaluated by monitoring changes in the susceptibility of sperm DNA to acid-induced denaturation *in situ* (Mean DFI and %DFI). Compared to the control group, statistically significant increases ($p<0.05$) in Mean DFI (as an index of sperm chromatin packaging) of 18 and 10%, were observed at 7 and 28 dpt, respectively (Figure 4A). In addition, 5.2- and 8-fold increases were observed in %DFI (an indicator of DNA integrity) at 7 and 28 dpt, respectively (Figure 4B). To confirm the genotoxic effect of Me-Pa, we evaluated the presence of sperm DNA nicks using *in situ*-nick translation (NT-positive cells) and we found 4.6- and 3.4-fold increases in the percentage of spermatozoa having DNA strand breaks at 7 and 28 dpt, respectively (Figure 4C). These data suggest that Me-Pa can alter both sperm chromatin condensation and DNA integrity after exposing maturing spermatozoa (7 dpt) and spermatocytes (28 dpt).

Me-Pa adversely affected in vitro-fertilization

Exposure to Me-Pa reduced the ability of mouse spermatozoa to fertilize cumulus-free oocytes collected from untreated females (Figure 5). When we analyzed zygotes at 8 h post-incubation, the percentage of fertilized oocytes decreased to 26% in Me-Pa-treated spermatozoa evaluated at 7 dpt, compared to 75% in the control group ($p<0.05$). A smaller but still statistically significant decrease (58% *vs* 75%, $p<0.05$) was observed in spermatozoa evaluated at 28 dpt as compared to the control group. This indicates a

differential sensitivity of epididymal spermatozoa and pachytene spermatocytes to the toxic effect of Me-Pa.

We then analyzed the cell division rate at 20 h after fertilization and found that the frequencies of embryos at the two-cell stage matched the frequencies of fertilized eggs observed at 8 h after fertilization for both treated groups (Figure 5). This suggests that the fertilized eggs were healthy enough to progress through the first cell division. Correlations between functional parameters such as MMP and induced-AR in sperm cells collected at 7 dpt were then performed, and found that the FR was lower as MMP and induced-AR decreased (Table 1), suggesting that both mitochondria and acrosome are target of Me-Pa in epididymal spermatozoa; whereas at 28 dpt only induced-AR correlated with decreased fertilization and cell division rates; suggesting that only the acrosome was affected by Me-Pa exposure.

Me-Pa adversely affected male fertility assessed by natural mating

The adverse effects of Me-Pa on sperm fertilizing capacity were observed also after natural matings. In fact, the *in vivo* FR of untreated females mated with Me-Pa-treated males was slightly but significantly reduced at 7 and 28 dpt (80% and 73%, respectively) as compared to the 89% of unexposed animals at at time 0 (Table 2). These results confirm that that damage caused by exposure to Me-Pa of epididymal spermatozoa (7 dpt) and spermatocytes (28 dpt) has consequences for their fertility potential.

Spermatozoa from Me-Pa-treated mice exhibited increased lipid peroxidation

Because OP toxicity has been recently associated with oxidative stress (Debnath and Mandal, 2000; Piña-Guzmán et al., 2006), and because sperm peroxidative damage by ROS may be involved in DNA fragmentation, membrane damage and impaired sperm function, we evaluated the induction of oxidative damage as a possible mechanism of Me-Pa action. To evaluate the relationship between Me-Pa exposure and LPO, spermatozoa from treated animals were examined for peroxidative damage by quantifying MDA production. LPO showed a significant increase (4-fold) in Me-Pa treated spermatozoa collected at 7 dpt (Figure 6), while no effect on MDA level was observed at 28 dpt with respect to control spermatozoa. To evaluate the correlation

between sperm fertilization potential and sperm function and oxidative stress, Spearman´s non-parametric statistics was applied. Highly significant negative correlations were observed between LPO and *in vitro* and *in vivo* FR, as well as, with MMP and induced-AR in spermatozoa collected at 7 dpt (Table 3). These results show that increased oxidative stress in mature spermatozoa affects their function and fertilization capacity (Table 4).

Discussion

There is growing concern about the potential for adverse reproductive effects of environmental toxicants, including pesticides such as OP that have demonstrated adverse effects on semen quality in humans and animals. Epidemiological studies have linked exposure to OP (including Me-Pa) with decreased semen quality, increased sperm DNA damage and hormonal imbalance (Padungtod et al., 2000; Recio et al., 2001, 2005; Sánchez-Peña et al., 2004). In addition, Me-Pa exposure caused testicular dysfunction (Narayana, 2006), reduced sperm quality (Mathew et al., 1992; Narayana et al., 2005), and increased sperm genetic damage (Narayana et al., 2005; Piña-Guzmán et al., 2006) in laboratory animals. The assessment of sperm quality is important but it is not the sole aspect for identifying and characterizing male reproductive toxicants. Therefore, more data are needed to evaluate the relationship between sperm functional parameters (including DNA integrity) and fertility potential. The present study investigated the function and fertilizing potential of spermatozoa from mice exposed to a single high dose of Me-Pa (20 mg/kg b.w)Me-Pa use is restricted in Mexico; however, it is widely used across the country and has been linked to accidental and suicidal poisonings (Durán-Nah and Collí-Quintal, 2000) in which people are exposed to 10- to 100-fold the mouse LD_{50}. Therefore, the high dose used in this paper, may represent doses achieved in accidental poisonings. In this study, we evaluated the effects in spermatozoa collected at 7 and 28 dpt to test the sensitivity of two different stages of spermatogenesis and oxidative damage was explored as a possible mechanism of Me-Pa toxicity. The main findings of our study are summarized in Table 5 and discussed below.

The current study showed that Me-Pa exposure impaired motility and viability in mature spermatozoa collected at 7 and 28 dpt, indicating that Me-Pa exposure had effects on both mature sperm and pachytene spermatocytes in agreement with previous results (Piña-Guzmán et al., 2006). Because it is known that sperm membrane function is important for sperm motility, metabolism, capacitation, and the ability to fuse with the oocyte, we examined mitochondrial function by evaluating MMP and found a significant decrease in this functional parameter in mouse spermatozoa collected at 7 dpt. This result indicates that Me-Pa impairs sperm mitochondrion during their transit through the

epididymis. Recent studies suggest that mitochondrion is an important early target for OP compounds resulting in altered ATP production and mitochondrial integrity in cultured neuronal cells (Massicotte et al., 2005). The lack of an effect on MMP at 28 dpt may be due to the fact that structural modifications of many organelles including mitochondria occur during spermatogenesis (Meinhardt et al., 1999); therefore, any change, such as decreased MMP, caused at earlier stages on the testis may not be present at the end of the epididymal maturation.

Acrosome integrity is essential for sperm to penetrate into the zona pellucida and fuse with the oocyte (Oura and Toshimori, 1990; Flesch and Gadella, 2000). In the same manner as acrosome integrity is important, the AR must be timed precisely. Premature or delayed AR will prevent sperm from penetrating the cumulus cells that surrounds the ovum (cumulus oophorus) and adhering to the zona pellucida (Bedford and Cross, 1999). In the present study, we evaluated AR induced by GABA, a ligand capable of mimicking the AR of sperm cells, representing a good predictor of fertilization success in physiological conditions (Shi et al., 1997). We found that mouse spermatozoa analyzed at 7 and 28 days after Me-Pa exposure had a significantly decreased ability to undergo the induced-AR. Furthermore, our data showed a significant increase in the number of spontaneously acrosome-reacted spermatozoa only at 28 dpt; this premature AR could be linked to the reduced number of sperm undergoing induced-AR in response to GABA at this time. This suggests that Me-Pa exposure may induce a structural disruption of the acrosome precursor organelle, the Golgi apparatus, in spermatocytes (Anakwe and Gerton, 1990). On the other hand, the effect of Me-Pa on induced-AR of spermatozoa collected at 7 dpt could be the result of acrosome membrane damage during the epididymal transit, where changes of membrane components occur (Jones, 1998).

Recent advances in understanding male infertility have implicated oxidative stress as a major causative factor (Aitken and Krausz, 2001). Sperm plasma membrane contains a characteristic high level of poli-unsaturated fatty acids (PUFA), which makes spermatozoa particularly susceptible to oxidative damage (Gravance et al., 2003). Results from the present study indicate that induced-AR, mitochondrial function (MMP), as well as the fertilizing potential of Me-Pa-exposed spermatozoa decreased at both times, and that the effects observed at 7 dpt were negatively correlated with LPO,

strongly suggesting that sperm alterations were related to oxidative damage. LPO was not detected in spermatozoa collected 28 dpt, as reported in our previous study (Piña-Guzmán et al., 2006), probably because of the important membrane changes that sperm cells undergo during the last steps of spermatogenesis and epididymal maturation (Jones, 1998) that may lead to a complete replacement of damaged membranes.

Oxidative stress has been related to exposure to several OP. In humans, Banerjee et al. (1999) observed increased levels of LPO and ROS scavenging enzymes in blood samples of OP-poisoned subjects, and Ranjbar et al. (2002) reported increased levels of erythrocyte LPO and reduced anti-oxidant capacity in OP pesticide manufacturing workers. *In vitro* exposure to chlorpyrifos resulted in the induction of erythrocyte LPO (Gultekin et al., 2000); while Sharma et al. (2005) reported that dimethoate exposure in rats increased the generation of free radicals in liver and brain, and Fortunato et al. (2006) showed that LPO in brain increased after acute malathion exposure in rats. Finally, Debnath and Mandal (2000) observed that quinalphos caused damage and degeneration of testicular tissue in rats due to free-radical mediated LPO.

The basis of oxidative stress caused by OP may be due either to their "redox-cycling" activity, where they generate superoxide anion and hence hydrogen peroxide, or via changes in normal antioxidant homeostasis. OP insecticides containing the P-S bond (called "thion") are converted to P-O (called "oxon") via the cytochrome P450 (CYP) system (Jokanovic, 2001). Bondy and Naderi (1994) reported that CYP-mediated activation induces the production of ROS through the redox cycling activity. Such finding supports the hypothesis that OP-induced male reproductive toxicity may arise from their *in situ* metabolism within the reproductive tract. OP bioactivation through CYP-mediated oxidative desulphuration, has been reported in brain (Albores et al., 2001) and testis (Soranno and Sultatos, 1992). Furthermore, preliminary results from our group revealed the presence of several CYP enzymes involved in Me-Pa metabolism in the male reproductive tract in mice (manuscript in preparation). In addition, the oxons *per se* are highly toxic compounds that account for the cytotoxic effects of OP; *in vitro* studies in human lymphocytes (Blasiak and Kowalik, 1999) and spermatozoa (Salazar-Arredondo et al., 2008) and mouse neuronal cells (Giordano et al., 2007) demonstrated the cytotoxic and genotoxic effects of oxons. Lipids and DNA, however, are not the only

targets for oxidative damage; proteins are also vulnerable to oxidative attack (Stadtman and Oliver, 1991). The ability of capacitated spermatozoa to fuse with oocytes depends on functional sperm plasma membrane proteins (Berger and Horner, 2003); if these proteins are oxidatively damaged, one might expect that sperm-oocyte interaction would also be affected. This study is focused on oxidative damage caused by Me-Pa, however, we can not discard that OP pesticides are also able to phosphorylate proteins, such as nuclear sperm protamines (Piña-Guzmán et al., 2005); therefore, Me-Pa may also alter sperm function through this mechanism since sperm capacitation and AR are processes regulated by protein phosphorylation (Grasa et al., 2009). Whether or not sperm proteins are phosphorylated or oxidatively altered by Me-Pa exposure deserves further study.

Fertilizing ability is an essential endpoint of male reproductive toxicity and gamete fertility potential is being considered for inclusion in routine *in vivo* mating trials (Berger et al., 2000). We observed diminished fertilizing potential in spermatozoa collected at 7 and 28 dpt; however, the underlying mechanisms seem to be different. During epididymal transit (7 dpt) sperm plasma membrane undergoes substantial remodeling (Dacheux et al., 2005) and mouse epididymal spermatozoa are characterized by high percentages of sphingomyelin and PUFA, mainly arachidonic, docosapentanoic and docosahexanoic acids that confer fluidity to sperm membranes (Rejraji et al., 2006). In addition, considering that most of sperm functions required for fertilization are developed in the epididymis, such as motility, zona binding and membrane fusion abilities (Cooper, 2007), it is not far fetched to hypothesize that a disturbed maturation of epididymal spermatozoa by Me-Pa exposure could impact their fertilizing capacity. On the other hand, as discussed earlier, the decreased fertilizing ability observed in sperm collected at 28 dpt might be related to alterations during the acrosome formation.

We observed Me-Pa-induced alterations of sperm chromatin condensation and DNA integrity after exposing spermatocytes (28 dpt) and maturing spermatozoa (7 dpt) in agreement with previous results (Piña-Guzmán et al., 2006). We can not discard the possibility that these sperm chromatin alterations may contribute to the decreased fertilization rates observed in this work, because DNA integrity and proper chromatin condensation are key for the fertilization process to occur (Sun et al., 1997). Studies conducted in humans suggest that values of NT-positive spermatozoa higher than 10%

are more frequent in those couples with problems in establishing a pregnancy (Tomlinson et al., 2001). In addition, Sun et al. (1997) reported that sperm samples with ≤4% DNA fragmentation (TUNEL) were associated with increased fertilization and embryo cleavage rates, while Duran et al. (2002) showed that no samples with >12% of TUNEL-positive spermatozoa resulted in pregnancy.

In this work, both *in vivo* and *in vitro* fertilization protocols clearly showed that exposure to a high dose of Me-Pa (20 mg/kg) affected the fertility of mouse spermatozoa. The *in vitro* FR in spermatozoa collected at 7 dpt was decreased at a higher level than in spermatozoa collected at 28 dpt. Results from the *in vivo* mating also showed a decreased FR in spermatozoa collected at both times, although more pronounced at 28 dpt. Comparing the FR from non-exposed mice, the value reached by the *in vivo* assay was higher than that obtained*in vitro* (89% vs 74%, $p<0.05$) in agreement with results from other authors who used similar IVF protocols (Cecconi et al., 1996; Torner et al., 1999). This could be explained by the optimal conditions found in the vagina where capacitation occurs, beside the presence of a number of first messengers in seminal plasma that regulate capacitation, and components that adequately protect spermatozoa against oxidative damage, all this could favor the fertilizing potential of ejaculated spermatozoa (Fraser et al., 2003). In summary, our results showed that acute Me-pa exposure induced membrane impairment, decreased motility and viability, caused DNA damage, mitochondrial and acrosome dysfunctions in mouse spermatozoa leading to reduced fertility evaluated by *in vitro* and *in vivo* assays. These effects were evident when either spermatocytes or maturing spermatozoa were exposed to Me-Pa suggesting that multiple male germ cell stages are sensitive to its action. The oxidative damage caused in sperm cells, probably due to Me-Pa metabolism, seems to be involved in these effects. Finally, our data suggest that the detrimental effects of occupational exposure to OP on male fertility may manifest immediately after exposure and persist for several months afterward as different germ cell stages can be affected.

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Conflict of Interest statement

The authors have disclosed having none potential conflict of interests regarding this manuscript.

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Table 1.

Correlation between functional sperm parameters and *in vitro* fertilization ability of sperm from Me-Pa-treated mice mated with control females.

Parameter	MMP		Induced-AR	
	7 dpt	28 dpt	7 dpt	28 dpt
Fertilization rate (8 h post-IVF)	0.8813*	-0.0250	0.9980*	0.7958*
Cell division rate (20 h post-IVF)	0.9676*	0.3948	0.9675*	0.9120*

Note. Values represent the correlation coefficients. MMP = mitochondrial membrane potential. AR = Acrosome reaction. dpt = days post-treatment. IVF = *In vitro* fertilization. * $p < 0.05$.

Table 2.

In vivo fertilization rates of Me-Pa-treated mice mated with control females.

Time of mating	n	Recovered eggs	Fertilized eggs	Fertilization rate (%)
Time 0	36	406	358	89.1 ± 4.2
7 days post-treatment	45	682	550	80.4 ± 1.3*
28 days pos- treatment	42	593	435	73.5 ± 2.9*+

Note. Values represent the sum of 3 independent experiments. n = number of females with vaginal plug. Fertilization rate: percentage of 1- and 2-cell embryos (mean ± SEM). * Compared to Time 0 and + compared to 7 days post-treatment ($p < 0.05$).

Table 3.

Correlation between LPO and sperm quality and functional parameters in Me-Pa exposed mice at 7 dpt.

Parameter	LPO
Viability	-0.5173*
Motility	-0.7053**
%DFI	0.6268*
NT-positive spermatozoa	0.9193**
MMP	-0.7172*
Induced-AR	-0.9490*

Note. Values represent the correlation coefficients. MMP = mitochondrial membrane potential. * p< 0.05. ** p< 0.0005. dpt = days post-treatment.

Table 4.

Correlation between LPO and fertilization rates in spermatozoa from Me-Pa exposed mice collected at 7 dpt.

Fertilization Assay	LPO
In vitro	-0.9472*****
In vivo	-0.8407*****

Note. Values represent the correlation coefficients. dpt = days post-treatment. * p< 0.05.

Table 5.

Differential effects of Me-Pa exposure on sperm quality, chromatin integrity, acrosome reaction, mitochondrial function, lipoperoxidation and fertilizing capacity of mice after 7 and 28 days-post treatment.

Parameter	7 dpt	28 dpt
Motility	▼	▼
Viability	▼	▼
Morphology	**NC**	▼
DNA damage	▲	▲
Spontaneous-AR	**NC**	▲
Induced-AR	▼	▼
MMP	▼	**NC**
LPO	▼	**NC**
In vivo FR	▼	▼
In vitro FR	▼	▼

▼ Denotes a decrease. ▲ Denotes an increase. NC = no change. AR = acrosome reaction. MMP = Mitochondrial membrane potential. LP = lipoperoxidation. FR = Fertilization rate. dpt = days post-treatment.

Figure legends

Fig. 1. Sperm quality parameters at 7 and 28 days after Me-Pa treatment of adult mice. Values are expressed as mean ± SD of at least 3 experiments by duplicate. *p < 0.05 compared to the control group according to the Student *t* test. n = 6 animals per group.

Fig. 2. Sperm mitochondrial membrane potential at 7 and 28 days after Me-Pa treatment of adult mice. Values are expressed as mean ± SD of at least 3 experiments by duplicate. *p < 0.05 compared to the control group according to the Student *t* test. MMP = mitochondrial membrane potential. n = 6 animals per group.

Fig. 3. Spontaneous- and GABA-induced acrosome reactions in spermatozoa from Me-Pa-treated mice at 7 and 28 days after exposure. Bars represent the mean ± SD of at least 3 experiments by triplicate. A total of 100 spermatozoa were scored for each treatment. C = Control group.T = Me-Pa-treated group. n = 6 animals per group.

Fig. 4. Chromatin structure and DNA integrity of mature spermatozoa from Me-Pa-treated mice at 7 and 28 days post-treatment. A) Mean DFI, B) %DFI, and C) NT-positive cells. Values are expressed as mean ± SD of at least 3 experiments by duplicate. *p < 0.05 compared to the control group according to the Student *t* test. n = 6 animals per group.

Fig. 5. *In vitro* fertilization rate in Me-Pa-treated male mice evaluated at 7 and 28 days post-treatment. Bars represent the mean ± SD of 3 independent microscopic observations. *Different from the control group and **different from the 8 h post-fertilization group (p < 0.05). dpt = days post-treatment. n = 6 animals per group.

Fig. 6. Effect of Me-Pa on lipidperoxidation in mouse spermatozoa. Values are expressed as mean ± SD of at least 3 experiments by duplicate. *p < 0.05 compared with the control group according to the Student *t* test. MDA= malondialdehyde. n = 6 animals per group.

Figure 1

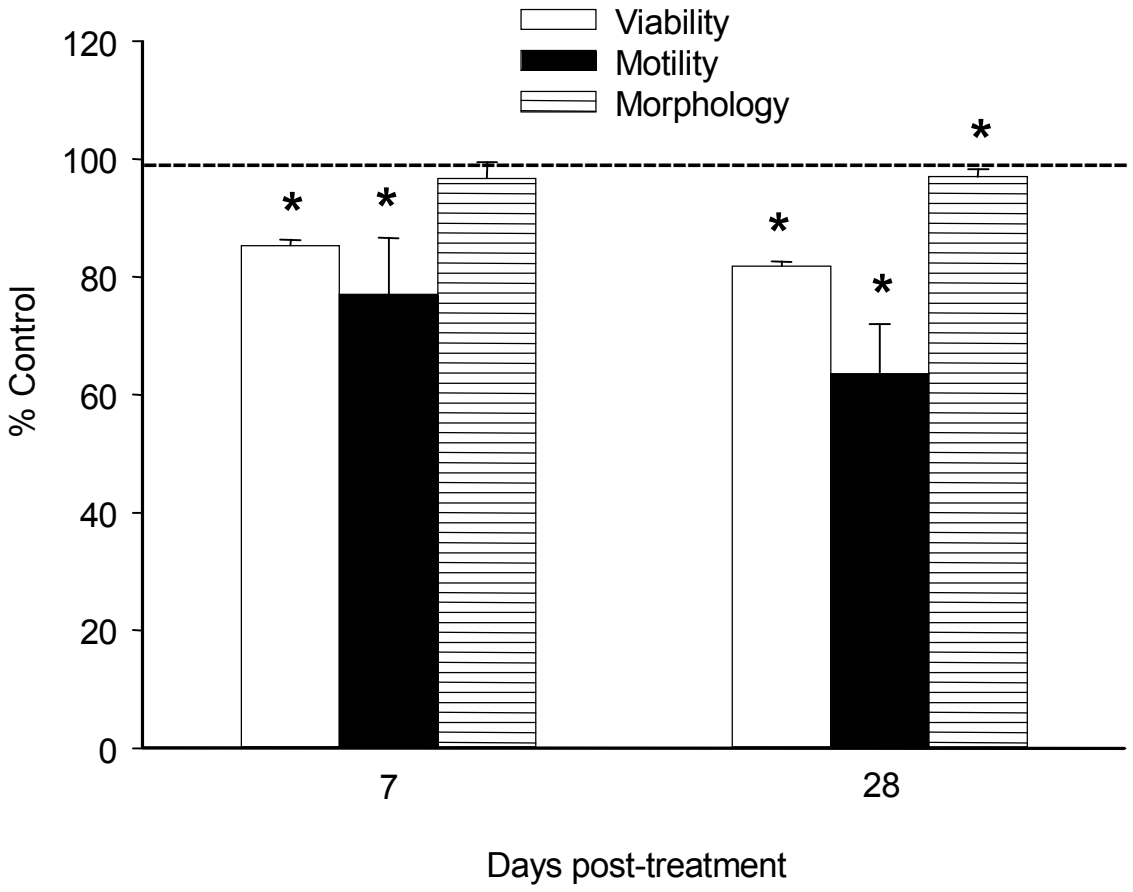

Viability
Motility
Morphology
120
100
80
60
40
20
0
% Control
*
*
*
*
*
7
28
Days post-treatment

Figure 2

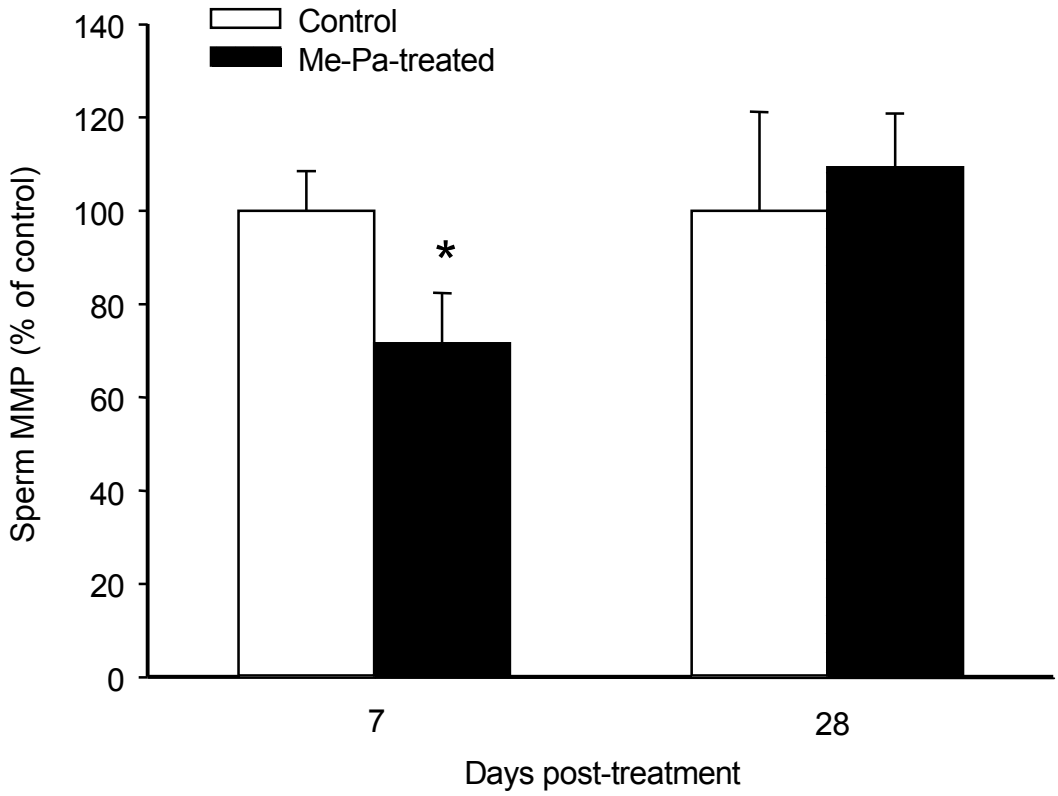

Control
Me-Pa-treated
140
120
100
80
60
40
20
0
Sperm MMP (% of control)
*
7
28
Days post-treatment

Figure 3

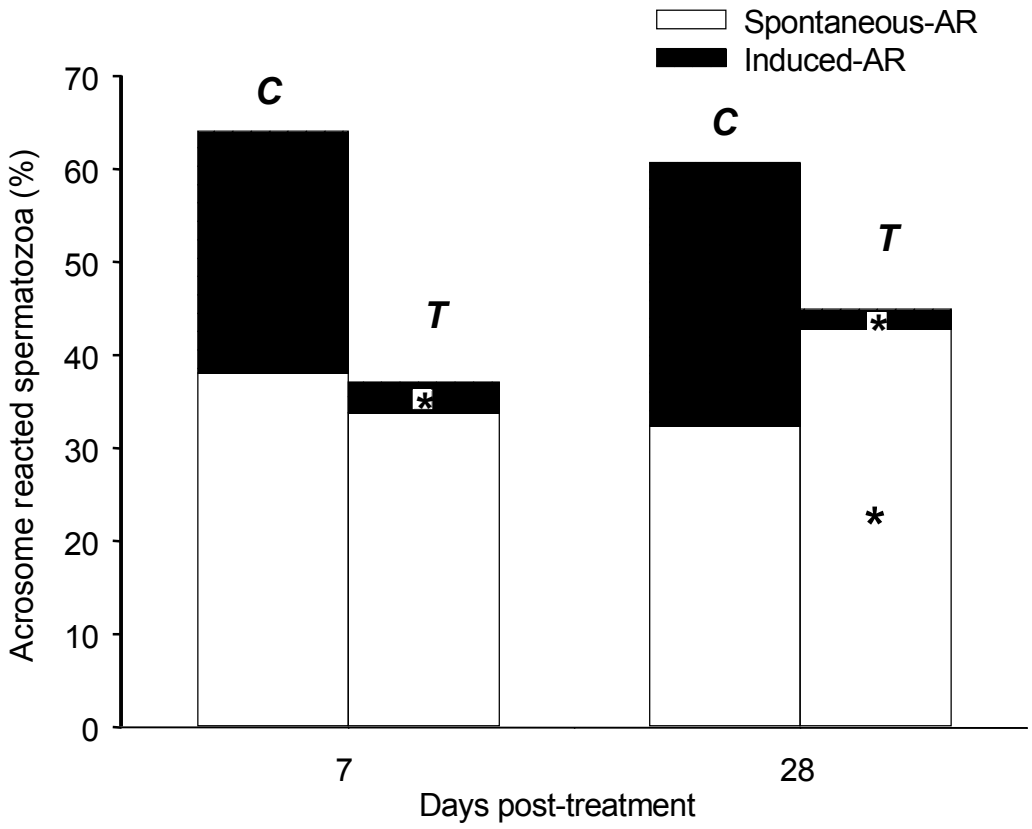

Spontaneous-AR
Induced-AR
Acrosome reacted spermatozoa (%)
70
60
50
40
30
20
10
0
C
T
C
T
*
*
*
7
28
Days post-treatment

Figure 4

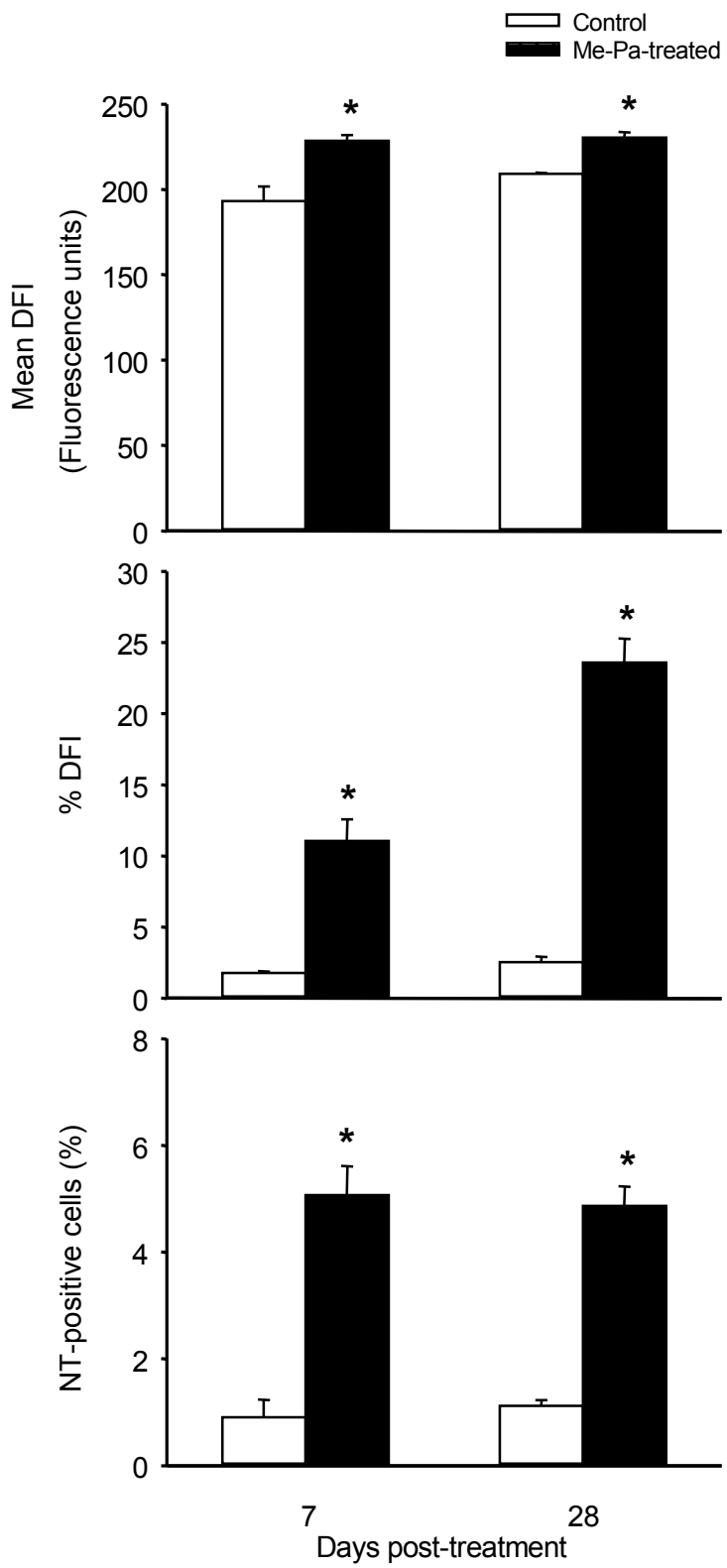

Control
Me-Pa-treated
Mean DFI
(Fluorescence units)
250
200
150
100
50
0
*
*
% DFI
30
25
20
15
10
5
0
*
*
NT-positive cells (%)
8
6
4
2
0
*
*
7
28
Days post-treatment

Figure 5

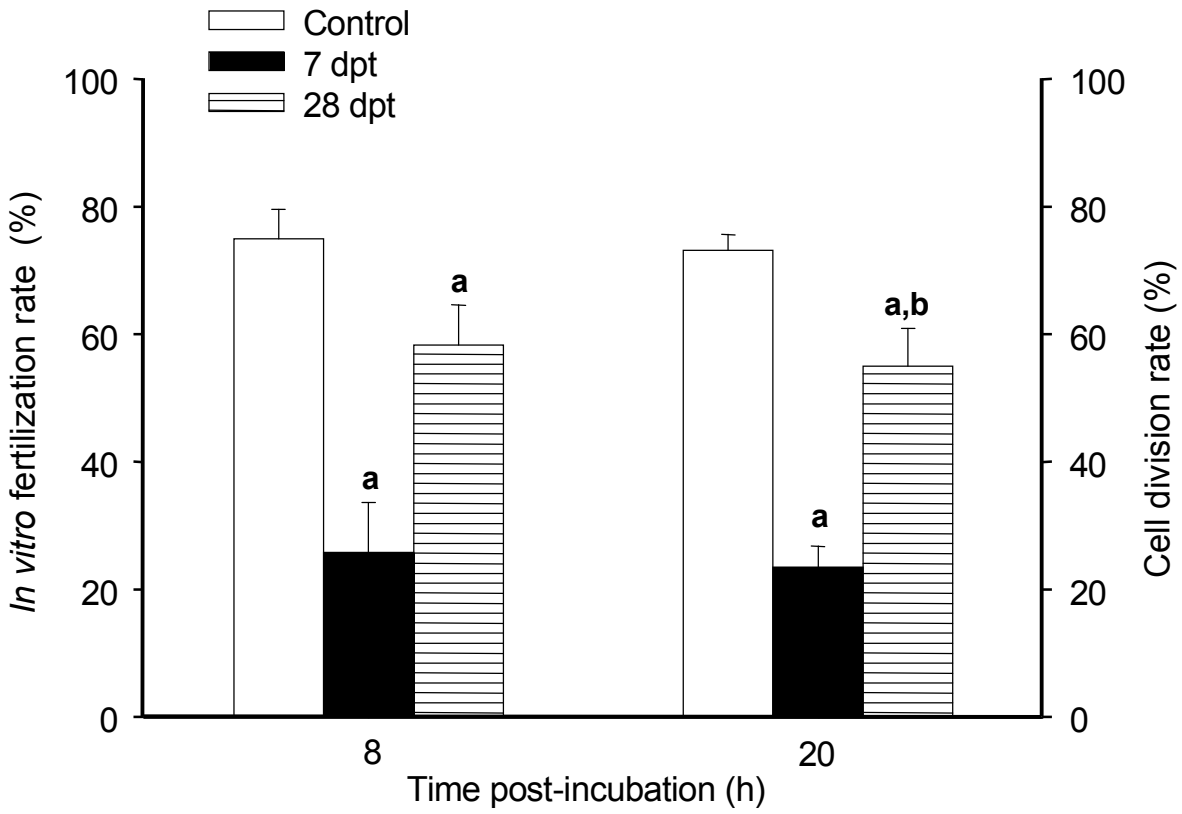
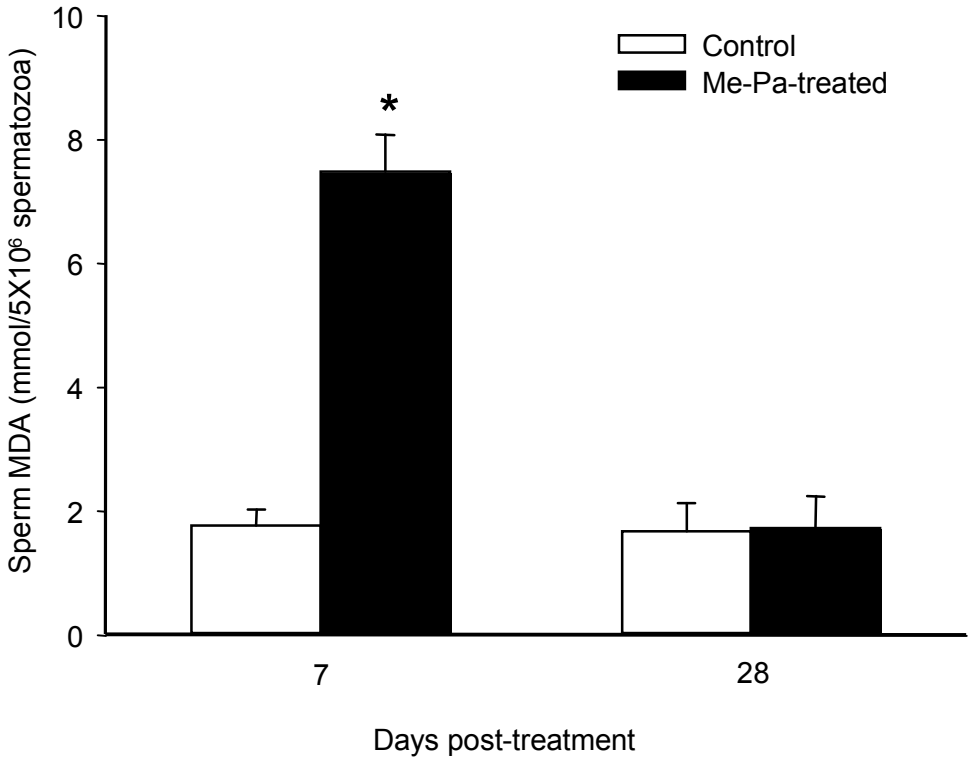

Control
7 dpt
28 dpt
100
80
60
40
20
0
In vitro fertilization rate (%)
Cell division rate (%)
a
a
a,b
8
20
Time post-incubation (h)

Figure 6


Control
Me-Pa-treated
*
Sperm MDA (mmol/5X10⁶ spermatozoa)
10
8
6
4
2
0
7
28
Days post-treatment