

Methamidophos alters sperm function and DNA at different stages of spermatogenesis in mice



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ARTICLE INFO

Article history:

Received 5 September 2013

Revised 14 June 2014

Accepted 22 June 2014

Available online 3 July 2014

Keywords:

Methamidophos

Sperm function

Male fertility

DNA damage

Organophosphate pesticides

Protein phosphorylation

ABSTRACT

Methamidophos (MET) is a highly toxic organophosphate (OP) pesticide that is widely used in developing countries. MET has male reproductive effects, including decreased fertility. We evaluated MET effects on sperm quality, fertilization and DNA integrity, exploring the sensitivity of different stages of spermatogenesis. Adult male mice received MET (3.75 or 5 mg/kg-bw/ip/day/4 days) and were euthanized 1, 28 or 45 days post-treatment (dpt) to evaluate MET's effects on epididymal maturation, meiosis or mitosis, respectively. Spermatozoa were obtained from the cauda epididymis–vas deferens and were evaluated for sperm quality, acrosome reaction (AR; Coomassie staining), mitochondrial membrane potential (by JC-1), DNA damage (comet assay), oxidative damage (malondialdehyde (MDA) production), in vitro fertilization and protein phosphorylation (immunodetection), and erythrocyte acetylcholinesterase (AChE) activity. At 1-dpt, MET inhibited AChE (43–57%) and increased abnormal cells (6%). While at 28- and 45-dpt, sperm motility and viability were significantly reduced with an increasing MET dose, and abnormal morphology increased at 5 mg/kg/day/4 days. MDA and mitochondrial activity were not affected at any dose or time. DNA damage (OTM and %DNA) was observed at 5 mg/kg/day/4 days in a time-dependent manner, whereas both parameters were altered in cells from mice exposed to 3.75 mg/kg/day/4 days only at 28-dpt. Depending on the time of collection, initial-, spontaneous- and induced-AR were altered at 5 mg/kg/day/4 days, and the fertilization capacity also decreased. Sperm phosphorylation (at serine and tyrosine residues) was observed at all time points. Data suggest that meiosis and mitosis are the more sensitive stages of spermatogenesis for MET reproductive toxicity compared to epididymal maturation.

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Introduction

The indiscriminate use of pesticides in developing countries is a public health problem. In Mexico, although underestimated, there are thousands of intoxication episodes per year (AMIFAC, 2013) and organophosphate (OP) pesticides are among the most frequently encountered substances in these cases. In the last two decades, several studies have reported that environmental or occupational exposure to pesticides is associated with decreased sperm quality (in the following order: concentration, motility and morphology) and OPs are the class of pesticides most frequently investigated (Martenies and Perry, 2013; Perry, 2008).

OP pesticides are widely used around the world, and methamidophos (MET; O,S-dimethyl phosphoramidothiolate), a highly toxic anticholinesterase pesticide, is one of the most used in agriculture and urban pest control in developing countries such as Mexico (Blanco-Muñoz et al., 2010; Pérez-Herrera et al., 2008; Rojas-García et al., 2011). Experimental and epidemiological studies have demonstrated that OP exposure alters male reproduction, including semen quality (Mathew et al., 1992; Padungtod et al., 2000; Recio et al., 2008), hormone levels (Padungtod et al., 1998; Recio et al., 2005; Yucra et al., 2006, 2008) and fertilization ability (Piña-Guzmán et al., 2009). OPs have also shown genotoxic effects on male germ cells (Padungtod et al., 1999; Recio et al., 2001; Sánchez-Peña et al., 2004) and have shown to induce oxidative damage as well (Piña-Guzmán et al., 2006; Sarabia et al., 2009). However, little is known about MET toxicity in mammalian reproduction. Burruel et al. (2000) reported an impairment of embryonic cell progression and an increase in the number of degenerated embryos after paternal exposure to MET (5 mg/kg-bw, intraperitoneal [ip]), whereas Farag et al. (2012) demonstrated that paternal MET exposure (2 and 3 mg/kg-bw/day/4 weeks, gavage) decreased the male fertility index, as shown by a

Abbreviations: AChE, acetylcholinesterase; AR, acrosome reaction; MET, methamidophos; MDA, malondialdehyde; OTM, olive tail moment; OP, organophosphate.
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decrease in pup survival and an increase in uterine resorptions. On the other hand, MET exposure (single and multiple ip injections of 4.5 and 6 mg/kg-bw) is associated with genetic changes in somatic cells, such as an increase in micronuclei in bone marrow, and the induction of sister chromatid exchange in mouse bone marrow cells in culture (Amer and Sayed, 1987). No literature is available about the effects of MET on germ cell DNA. We suggested that exposure to OPs alters the chromatin structure and produces DNA strand breaks in mouse spermatozoa and that these changes may result in DNA mutations that alter embryonic development or predispose the offspring to a risk of developmental alterations or pathologies such as cancer (Piña-Guzmán et al., 2005, 2006).

Previous studies have shown that sperm cells at different stages of spermatogenesis are the target of OP pesticide exposure, particularly their nuclei. Piña-Guzmán et al. (2006, 2009) showed that methylparathion (Me-Pa) caused dose-related alterations in the integrity of chromatin and DNA, mitochondrial membrane potential (MMP) and acrosome reaction (AR), as well as oxidative damage and reduced fertilization ability in sperm cells collected at 7 and 28 days post-treatment (dpt) as a reflection of the damage to mature spermatozoa and spermatocytes, respectively. These data suggest that both stages of spermatogenesis were sensitive to Me-Pa. Similarly, diazinon showed nuclear protein phosphorylation and chromatin alterations in sperm cells collected at 8-dpt but not at 15-dpt, suggesting that the last step of spermatid differentiation is the sensitive stage of OP toxicity (Piña-Guzmán et al., 2005). However, the fungicide carbendazim appears to target pachytene spermatocytes and step 14 spermatids at the time of first exposure (Kadalmani et al., 2002).

Three main mechanisms of OP toxicity have been studied: i) phosphorylation of proteins, such as sperm protamines by diazinon exposure (Piña-Guzmán et al., 2005); ii) oxidative damage as indicated by the presence of the oxidized adduct 8-hydroxydeoxyguanosine (8-OHdG) in spermatozoa from mice exposed to Me-Pa (Monroy-Peréz et al., 2012), and iii) alkylation mainly of DNA as shown by the formation of [7-¹⁴C] methylguanaine in mouse liver cells after exposure to [OCH₃-¹⁴C]MET (Zayed and Mahdi, 1987).

Therefore, we evaluated the acute effects of different concentrations of MET on sperm function, DNA integrity and fertilization capacity evaluating the sensitivity of cells at different stages of spermatogenesis in adult mice. Furthermore, to explore the mechanisms underlying MET toxicity on male reproduction, we evaluated the ability of MET to cause oxidative damage and to phosphorylate sperm proteins.

Methods

Chemicals. Technical grade methamidophos (MET; 99.9% purity) was from Chem Service (West Chester, PA). Acetylthiocholine iodide, dithio-bis nitrobenzoic acid (DTNB), low melting point agarose (LMPA), normal melting point agarose (NMPA), ethidium bromide, sodium orthovanadate, bovine serum albumin (BSA), Ponceau S red, Tween-20, dodecyl sodium sulfate (SDS), penicillin, streptomycin, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) were from Sigma Chemical Company (St. Louis, MO). Acrylamide, bis-acrylamide, protease inhibitors (MiniComplete), nitrocellulose membranes and molecular weight markers were from Roche Applied Science (Mannheim, Germany). The chemiluminescence kit was from Kodak GBX (Rochester, NY) and the LPO-FR 12 assay kit was from Oxford Medical Research (Oxford, MI). Gamma aminobutyric acid (GABA) was from USB Amersham Life Science (Amersham, UK), and JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl carbocyanine iodide) and carbonyl cyanide m-chlorophenylhydrazine (CCCP) were from Molecular Probes (Invitrogen, Mexico). Antibodies anti-rabbit anti-phosphotyrosine and anti-rabbit anti-phosphoserine and the anti-rabbit secondary antibody coupled to horse radish peroxidase were also from Invitrogen (Mexico). Anti-actin primary antibody was donated by Dr. Manuel Hernández-Hernández from the Department of Cell Biology (CINVESTAV-IPN). Coomassie blue-G250 and

Trypan blue were from Mallinckrodt (Mexico), and formaldehyde and Papanicolaou OG-6 staining were from Merck (Darmstadt, Germany). Cytoseal-60 mounting medium was from Pellico International (Ted Pella, Inc., CA). All other reagents were of chemical grade of the highest quality.

Animals and treatment. Adult male ICR-CD1 mice (10–12 weeks old) were obtained from our institutional animal facility and housed in filtered cages, maintained under 12-h dark–light cycles, with food and water available ad libitum. MET was dissolved in saline solution (NaCl 0.9%) and administered via ip at doses of 3.75 and 5 mg/kg/day for 4 days (time reported for the completion of mice epididymal maturation) (Peirce and Breed, 2001). Doses were selected based on the LD₅₀ reported by Burrueal et al. (2000), which is 10–15 mg/kg (ip); therefore, doses represent 1/4 or 1/3 if taking 15 mg/kg as the DL₅₀. The exposure time (4 days) was chosen to ensure the exposure to mature cells because the epididymal maturation (a candidate target stage) of mice spermatozoa is completed in approximately 4–6 days (Peirce and Breed, 2001). Controls received the vehicle only (0.1 ml saline). Six animals were dosed with MET and 4 were given saline (control group) per dose and per time of cell collection (see below); 2 independent experiments were performed. After dosing, animals were observed for up to 12 h for common cholinergic symptoms. All animal procedures were approved by the Institutional Animal Care and Use Committee (CICUAL) in compliance with the International Guidelines for the use and care of laboratory animals.

Sperm isolation and analysis. Mice were euthanized 1, 28 or 45 days after the last administration of MET (days post-treatment, dpt), corresponding to cells that were at the stages of epididymal maturation, meiosis or mitosis, respectively, at the time of the exposure. Cauda epididymis and vas deferens (CE-VD) were excised and freed of the fat pad, blood vessels, and connective tissue and spermatozoa were flushed with saline solution. Spermatozoa were analyzed by light microscopy according to the WHO guidelines (2010), including sperm concentration using a hemocytometer, the percentage of viable cells using 0.5% Trypan blue, progressive motile cells by phase contrast microscopy (as the percentage from 200 sperm cells) and sperm morphology following the Papanicolaou-modified staining technique and the scoring and classification described by Wyrobek et al. (1983).

Sperm comet assay. The DNA damage was evaluated using the comet assay according to Singh et al. (1988) with some modifications. Briefly, slides prepared with the sperm suspension were coated with a microgel formed with 0.5% NMPA, and then cells (1×10^4 sperm cells/ml) were suspended in 75 μ l of 0.5% LMPA (w/v), poured on the microgel, and chilled at 4 °C for 15 min; immediately, a second layer of 0.5% LMPA (w/v) was poured. Slides were dipped in cell lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 40 mM DTT, pH 10) at 4 °C for 2 h protected from light. After lysis, slides were placed in running buffer (300 mM NaOH, 10 mM EDTA, pH 13) for 20 min. Finally, electrophoresis was performed at 4 °C, 25 V and 300 mA for 25 min. Samples were run in duplicate. After electrophoresis, slides were stained with ethidium bromide and images were taken randomly from 50 cells/slide and digitally captured for the analysis using the Comet Score™ v. 1.5 software. The percentage of DNA in the tail (%DNA) and the Olive tail moment (OTM) were calculated as previously reported (Olive, 1999).

Lipid peroxidation. We evaluated lipid peroxidation (LPO) by means of malondialdehyde (MDA) production using the LPO-FR 12 assay kit according to the manufacturer's protocol for cell lysates. An aliquot of sperm suspension containing 5×10^6 sperm cells/ml was used, incubated at 46 °C for 1 h, and analyzed at 586 nm. Volumes were adjusted for a 96-well microplate assay. Sperm cells from a control animal were

incubated with 30 mM H₂O₂ at 37 °C for 1 h as a positive control for MDA production.

Acrosome reaction (AR). The percentage of acrosome-reacted spermatozoa (without the acrosome) was determined using Coomassie blue G-250 (Thaler and Cardullo, 1995). Briefly, sperm samples were incubated for capacitation in M16 medium (100 mM NaCl, 25 mM NaHCO₃, 5.5 mM glucose, 2.6 mM KCl, 1.5 mM Na₂HPO₄, 0.5 mM sodium pyruvate, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 20 mM sodium lactate, 100 IU/mg penicillin, 100 g/ml streptomycin, pH 7.4) supplemented with 4 mg/ml BSA for 0 or 60 min to evaluate the initial- or spontaneous-AR, respectively. To evaluate the induced-AR, sperm cells were capacitated for 60 min and then treated with 0.5 μM GABA for 15 min (GABA functions as a putative modulator of sperm function that triggers the AR) (Shi et al., 1997). Cells were fixed in 10% formaldehyde in PBS (pH 7.5) for 10 min followed by centrifugation at 9000 rpm/1 min. The pellet was washed, dissolved in ammonium acetate (0.1 M, pH 9.0), centrifuged and suspended in 20–50 μl PBS. Cells were then spread on glass slides, air-dried and stained with 0.22% (w/v) Coomassie blue-G250 in 50% methanol and 10% glacial acetic acid for 2 min; slides were gently rinsed with deionized water, air dried, and mounted with Cytoseal-60 mounting medium. To calculate the percentage of AR, at least 200 cells per sample were randomly scored and examined by phase-contrast microscopy at a magnification of 100×. Cells were classified as having an intact acrosome (dark blue staining of the acrosome vesicle) or acrosome-reacted (no staining in the acrosome region). Results are expressed as the percentage of acrosome-reacted spermatozoa.

Mitochondrial membrane potential (MMP). Sperm mitochondrial activity was evaluated using the lipophilic cationic dye JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl carbocyanine iodide), which selectively enters healthy mitochondria with high transmembrane potential and accumulates as aggregates that emit a red fluorescence (Reers et al., 1991). In damaged mitochondria in which the trans-membrane potential is lost, the dye remains in the cytoplasm in a monomeric form emitting a green fluorescence. Briefly, sperm samples containing 1 × 10⁶ cells/ml were incubated with 2 μM JC-1 for 15 min at 37 °C in a high humidity incubator under 5% CO₂. As a positive control, cells were incubated with the proton gradient uncoupler CCCP (5 μM). Immediately after incubation, samples were analyzed for red and green fluorescence (FACSort, Becton Dickinson). A total of 5000 gated events were analyzed per sample at a flow rate of 100–300 events/s. A 488 nm filter was used for the excitation of JC-1 and 535 nm (green) and 595 nm (red) emission filters were used for detection. Results are expressed as the ratio of green/red fluorescence.

Oocyte collection and insemination for in vitro fertilization (IVF). For oocyte collection, female ICR-CDI mice (6 weeks old) were induced for superovulation via an ip injection of 7.5 IU of eCG followed by 5 IU of hCG 48 h later. Females were euthanized 16 h after the hCG injection by cervical dislocation. Uterine ovary-oviduct complexes were dissected and suspended in M16 medium supplemented with 4 mg/ml BSA, the ampulla was then punctured, and the cumulus-oocyte complex was extruded and placed in M-16 medium supplemented with 4 mg/ml of BSA; oocytes were washed with medium and then incubated at 37 °C under 5% CO₂ until use. Approximately 15–20 oocytes were obtained per female.

For oocyte insemination, spermatozoa collected from animals treated with the highest dose (5 mg/kg/day/4 days) were incubated in capacitating medium (M16 supplemented with 4 mg/ml BSA) until spontaneous-AR was observed (1 h at 37 °C, 5% CO₂). To assess fertilization, 15–20 oocytes suspended in 300 μl M16 medium were inseminated with 1 × 10⁶ cells (in 10 μl) of capacitated spermatozoa from control or MET-treated mice (5 mg/kg/day/4 days) in a standard slide with a spherical depression of approximately 0.5–0.8 mm depth. Gametes

were co-incubated for 36 h in a humidified incubator at 37 °C and an atmosphere of 5% CO₂ 95% air. Sperm-oocyte complexes were gently rinsed with M16 medium, fixed 1:1 (v/v) in 3% formaldehyde and observed by phase contrast microscopy. Oocytes were considered fertilized when 2- to 4-cell embryos were detected.

Phosphorylation of sperm proteins. To further evaluate the degree of phosphorylation in whole cells, spermatozoa collected from animals treated with the highest dose (5 mg/kg/day/4 days) were subjected to protein extraction. Aliquots of 10 × 10⁶ spermatozoa from control and MET-treated mice were incubated with lysis solution (125 mM Tris-HCl, pH 7.5, 3% SDS, 1 mM Na₃VO₄, 1 mM NaF) and a protease inhibitor cocktail (MiniComplete) for 30 min at 4 °C, and then sonicated for 1 min at 10 s intervals. Immediately, cell homogenates were centrifuged at 4000 rpm for 6 min and supernatants were kept in aliquots at –70 °C until use.

For the evaluation of protein phosphorylation, 50 μg of total protein (sperm homogenate) from cells from control and MET-treated mice were resolved by SDS-PAGE electrophoresis in 12% gels (120 v) and transferred to nitrocellulose membranes in 25 mM Tris-base, 192 mM glycine, pH 8.3 and methanol (v/v) at 400 mA. Transfer efficiency was checked by Ponceau S red (0.05%) staining. Membranes were then incubated with either anti-rabbit anti-phosphoserine (1:1000 dilution) or anti-rabbit anti-phosphotyrosine (1:2000 dilution) antibodies overnight at 4 °C with moderate shaking, followed by incubation with an anti-rabbit secondary antibody coupled to horseradish peroxidase (1:5000) for 1 h at room temperature. Non-specific binding was blocked by incubation in 50 mM Tris, 150 mM NaCl, pH 7.6, containing 0.1% Tween-20 and 1% BSA for 1 h at room temperature. Actin was used as the control. Membranes were washed and phosphorylated proteins were detected using an enhanced chemiluminescence kit according to the manufacturer's instructions. The densitometry analysis was performed by scanning images using the GelPro Analyzer Media Cybernetics software (Silver Spring, MD, USA), and normalized for actin content.

Acetylcholinesterase activity. Erythrocyte AChE activity was determined in whole blood by measuring the hydrolysis of acetylthiocholine iodide according to the method of Ellman et al. (1961). Peripheral blood samples were obtained by cardiac puncture 24 h after the last administration of MET and were collected in vials containing heparin. Thiocholine iodide and 5-dithiobis-2-nitrobenzoic acid (DTNB) containing quinidine sulfate were used as substrate and inhibitor of plasma cholinesterases, respectively. Samples were analyzed at 412 nm for 6 min at room temperature.

Statistical analyses. Data from two independent experiments are presented as the median, 75th percentile and maximum and minimum or the median and range, as appropriate. Data from both experiments were not significantly different, therefore, values were taken together for statistical analyses (n = 8 controls and n = 12 treated animals). Comparisons among groups (time of collection) were performed using the Kruskal-Wallis test, and comparisons between doses were performed using the non-parametric Wilcoxon U test. Dose- and time-dependent analyses were performed using linear regression. All data were analyzed using the STATA software version 12.0 (STATA Corp., College Station, TX). For all comparisons, statistical significance was assigned at p < 0.05.

Results

Body and relative organ weights

The body and relative organ weights in liver, kidneys and testes significantly decreased at 1-dpt at both doses of MET (Table 1); however,

they recovered at 28- and 45-dpt, except for testes weight, which was significantly lower at 45-dpt in mice exposed to both doses of MET.

Acetylcholinesterase inhibition after MET treatment

The inhibition of erythrocyte AChE activity, as an indicator of general toxicity (Lionetto et al., 2013), was evaluated 24 h after the last administration in all exposed groups (for cell collection at 1-, 28- and 45-dpt). The AChE activity showed an inhibition that correlated with MET doses; 46% and 58% inhibition were observed at 3.75 mg/kg/day/4 days and 5 mg/kg/day/4 days, respectively, compared to the control groups (Data not shown). The recovery of cholinergic symptoms was observed approximately 9 h after MET administration.

Effects of MET exposure on sperm quality

Sperm quality parameters showed the capacity of MET to alter male germinal cells (Fig. 1). At 1-dpt, MET did not alter sperm concentration or viability, but slightly decreased normal morphology and increased sperm motility at the highest dose of 5 mg/kg/day/4 days. However, sperm cells collected at 28- and 45-dpt were more sensitive to MET effects. Motility and viability decreased in a dose-dependent manner (Table 2), whereas normal morphology decreased only at the highest dose at both collection time points (Fig. 1). Stronger time-dependent effects on sperm parameters were observed at the dose of 5 mg/kg/day/4 days (Table 3). No interaction between dose and time of collection was observed in sperm parameters (see Table 5 in the Supplementary material). Sperm concentration was not altered at any time point. These results indicated that acute MET exposure alters sperm cells at early stages of spermatogenesis.

Effects of MET exposure on sperm membrane and mitochondrial function

Peroxidative damage (MDA production) in sperm cells from mice treated with MET was assessed as a possible mechanism underlying sperm damage. Sperm cells from MET-treated mice with 5 mg/kg/day/4 days and evaluated at 1-, 28- or 45-dpt showed similar MDA levels to control mice, indicating no peroxidative damage ($p > 0.05$) (Table 4). On the other hand, the loss of the MMP is a sensitive indicator of mitochondrial damage (Wang et al., 2003), therefore, we evaluated the MMP using the fluorophore JC-1 and observed no changes in the membrane potential of spermatozoa from mice treated with 5 mg/kg/day/4 days at 1-dpt nor at the other times of collection (Table 4). This may be due to important changes that membranes from sperm cells undergo during the final stages of spermatogenesis and epididymal maturation (Jones, 1998) that may lead to a complete replacement of damaged membranes as previously observed with other OPs (Piña-Guzmán et al., 2009).

Effects of MET exposure on the sperm acrosome reaction

We evaluated the ability of MET-treated and untreated spermatozoa collected at all time points (1-, 28- and 45-dpt) to undergo AR with oocytes obtained from untreated superovulated females. The percentage of spermatozoa undergoing AR was evaluated at 0 (initial-AR) and 1 h (spontaneous-AR) of capacitation, and after GABA incubation for 15 min (induced-AR) (Fig. 2). Mice treated with the high dose (5 mg/kg/day/4 days) showed alterations in spontaneous- and induced-AR, showing time-dependent patterns; very slight effects were observed in the initial-AR (Table 3). Cells collected at 45-dpt were the most sensitive, with increases of 49, 67, and 51% in the spontaneous-AR at 1-, 28- and 45-dpt, respectively; whereas decreases of 26, 29 and 41% were observed in the induced-AR at 1-, 28-, and 45-dpt, respectively, compared to the control groups. No interaction between dose and time of collection was observed in the AR (see Table 5 in the Supplementary material).

Effects of MET exposure on sperm DNA integrity

To evaluate the ability of MET to induce DNA damage, sperm cells were assessed by the comet assay, a useful tool for detecting DNA strand breaks, and expressed as %DNA and OTM (Singh et al., 1988). At 1-dpt, there was an increase in %DNA (1.7-fold) and OTM (5-fold) values only at the dose of 5 mg/kg/day/4 days, compared to the control group (Figs. 3a–b), whereas dose-dependent increases were observed at 28-dpt in both %DNA (1.4 to 3-fold) and OTM (7 to 12.6-fold) (Figs. 3a–b, Table 2). At 45-dpt cells from mice exposed to the highest dose (5 mg/kg/day/4 days) showed DNA damage, with increases of 1.3-fold in %DNA and 2-fold in OTM values. Finally, the time of cell collection significantly influenced the genotoxic effects of MET (%DNA and OTM parameters) at the dose of 5 mg/kg/day/4 days (Table 3). No interaction between dose and time of collection was observed in the DNA damage (see Table 5 in the Supplementary material).

Effects of MET exposure on IVF

We assessed the sperm function as the ability to fertilize an oocyte in vitro in cells that were collected at different time points after treatment to the highest dose of the pesticide (5 mg/kg/day/4 days); oocytes from females that had not been treated with MET were used for this purpose. A time-dependent decrease was observed in the capacity of spermatozoa to fertilize oocytes, with no effect observed in cells collected at 1-dpt (Fig. 4, Table 3). At 28-dpt, a decreased fertilization ability (18% reduction) was observed compared to the control group (76 ± 4.7 vs. 63 ± 4.4), whereas the strongest effect (31% reduction) was observed in cells collected at 45-dpt (77 ± 3.7 vs. 53 ± 1.9) (Fig. 4). No interaction between dose and time of collection was observed in the IVF ability (see Table 5 in the Supplementary material).

Table 1
MET exposure alters the body and relative organ weights at 1-, but not at 28- or 45-dpt.

Group	Body weight (g)	Liver (%)	Kidney (%)	Testes (%)	Seminal vesicles/ coagulating glands (%)	Prostate (%)
MET (mg/kg/day/4 days/1-dpt)						
Control	43.07 (37.8–50.0)	6.09 (5.3–6.6)	1.38 (1.2–1.7)	0.25 (0.2–0.3)	0.89 (0.7–1.6)	0.024 (0.021–0.052)
3.75	34.46 (31.3–39.5)*	5.30 (5.1–5.5)*	1.12 (0.9–1.4)*	0.22 (0.2–0.3)*	1.03 (0.9–1.2)	0.021 (0.021–0.025)
5	38.79 (35.3–43.4)*	5.18 (3.8–6.2)*	1.18 (0.7–1.3)*	0.23 (0.2–0.23)*	0.88 (0.7–1.4)	0.022 (0.020–0.024)
MET (mg/kg/day/4 days/28-dpt)						
Control	44.21 (41.1–50.1)	6.26 (5.4–7.0)	1.51 (1.2–1.8)	0.59 (0.5–0.7)	0.92 (0.7–1.3)	0.026 (0.025–0.029)
3.75	38.72 (36.3–41.6)	6.05 (5.0–6.7)	1.43 (1.3–1.5)	0.69 (0.5–0.9)	0.85 (0.7–0.9)	0.026 (0.026–0.03)
5	39.11 (35.3–45.4)	5.42 (5.1–6.1)	1.50 (1.3–1.8)	0.58 (0.5–0.9)	1.02 (0.9–1.3)	0.028 (0.026–0.03)
MET (mg/kg/day/4 days/45-dpt)						
Control	44.17 (49.6–49.6)	5.96 (5.7–6.2)	1.52 (1.3–1.7)	0.79 (0.7–0.9)	0.94 (0.8–1.8)	0.025 (0.024–0.029)
3.75	42.4 (39.8–43.3)	5.89 (4.9–6.9)	1.73 (1.4–1.9)	0.50 (0.2–0.7)*	0.94 (0.7–1.3)	0.023 (0.023–0.025)
5	42.9 (41.2–49.5)	6.08 (5.2–6.9)	1.72 (1.5–1.8)	0.71 (0.6–0.8)*	0.94 (0.9–1.9)	0.025 (0.023–0.039)

Data are expressed as median and range ($n = 8$ controls; $n = 12$ treated animals per dose) from two independent experiments. *Significant difference ($p < 0.05$) compared to control mice according to the non-parametric Mann–Whitney test.

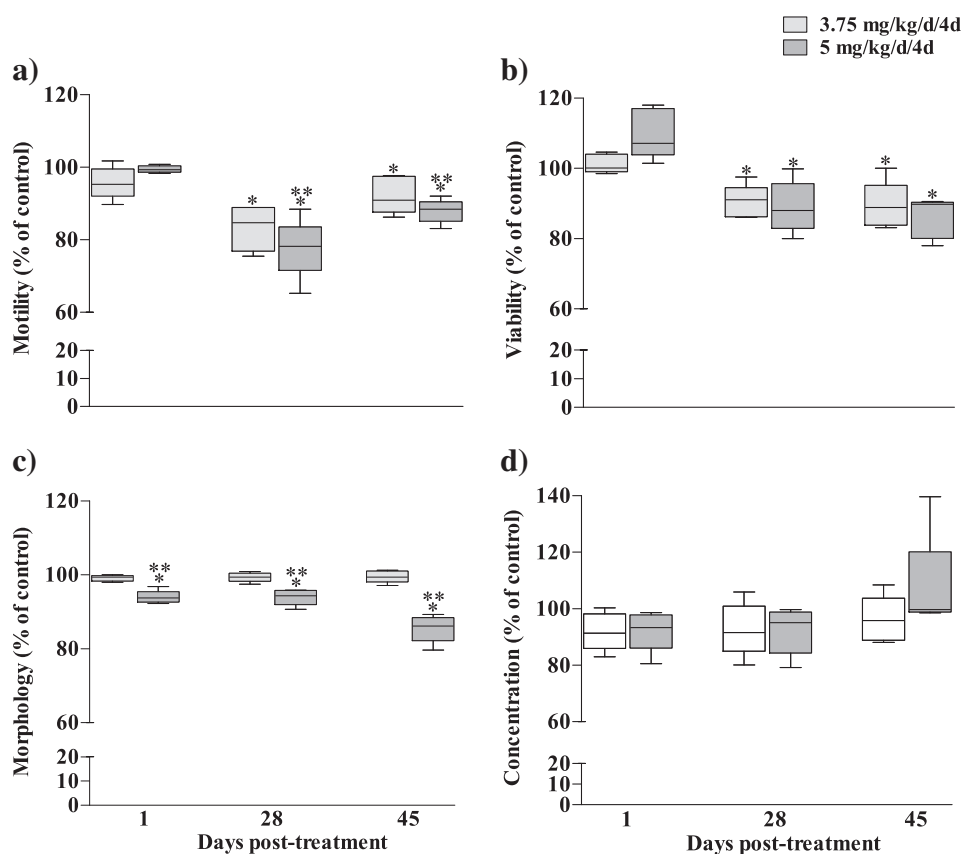


Fig. 1. Sperm quality parameters after MET exposure in mice. Spermatozoa were collected at 1-, 28- or 45-dpt and evaluated for motility (a), viability (b), morphology (c), and concentration (d). Data represent the median, 75th percentile and maximum and minimum (n = 8 controls and n = 12 treated mice per dose). *Significant difference compared to control mice (p < 0.001) according to the Wilcoxon U test. **Significant difference compared to the 3.75 mg/kg/day group (p < 0.05) according to the Kruskal–Wallis test.

Based on the absence of significant results in sperm quality and AR at 1-dpt at the dose of 3.75 mg/kg/day/4 days, we decided to evaluate the sperm fertilization ability at 28- and 45-dpt using only the highest dose of 5 mg/kg/day/4 days to reduce the number of animals.

Sperm protein phosphorylation by MET exposure

We evaluated the ability of MET to phosphorylate proteins in the total homogenate of spermatozoa collected at all time points after exposure to the highest dose (5 mg/kg/day/4 days), using anti-phosphoserine or anti-phosphotyrosine antibodies. Densitometry analyses showed increases in the phosphorylation of both residues, compared with the corresponding control group, with sperm cells exposed to MET at testicular stages (28-

and 45-dpt) being the most affected, particularly at serine residues (Fig. 5, Table 3). No interaction between dose and time of collection was observed in the phosphorylation levels (see Table 5 in the Supplementary material). At 1-dpt, increases of 27 and 21% in the phosphorylation of serine and tyrosine residues, respectively, were observed, with an increased signal in bands of approximate molecular weights between 27 to 73 kDa and 39 to 130 kDa, respectively (Figs. 6a and d). At 28-dpt, the increases in protein phosphorylation were slightly higher, 41 and 35% for serine and tyrosine residues, respectively (Fig. 5), in bands of 15 to 58 kDa and 40 to 110 kDa, respectively (Figs. 6b and e). At 45-dpt, the highest increase

Table 2
Dose–response relationship of sperm parameters evaluated in mice exposed to MET.

Parameter	Correlation coefficient		
	1-dpt	28-dpt	45-dpt
Sperm quality			
Motility	−0.308	−4.364**	−2.452**
Viability	1.714**	−2.281*	−2.545**
Morphology	−1.245**	−1.334**	−2.749**
Concentration	−0.314	−0.223	1.946
Acrosome reaction			
Initial-AR	−1.117**	0.573	0.686*
Spontaneous-AR	2.744**	4.098**	4.581**
Induced-AR	−2.214**	−2.241*	−2.915**
DNA damage (comet assay)			
OTM	4.903**	6.284**	6.489**
%DNA	3.521**	4.730**	4.508**

Animals were exposed to 3.75 or 5 mg/kg/day/4 days, and sperm cells were collected and analyzed at 1-, 28- or 45-dpt. * p < 0.010; and **p ≤ 0.001. dpt = days post-treatment.

Table 3
Time–response relationship of sperm parameters evaluated in mice exposed to MET.

Parameter	Correlation coefficient	p value
3.75 mg/kg/day/4 days		
Motility	−0.2889	0.095
Viability	−0.3095	0.001
OTM	0.1390	0.066
%DNA	0.1078	0.054
5 mg/kg/day/4 days		
Motility	−0.9074	0.001
Viability	−0.5236	0.001
Morphology	−0.3165	0.001
Initial AR	0.1245	0.001
Spontaneous AR	0.2296	0.001
Induced AR	−0.2611	0.001
OTM	0.2746	0.019
%DNA	0.2087	0.001
IVF	−0.2716	0.015
Phosphorylation on tyrosine	0.0004	0.998
Phosphorylation on serine	1.5802	0.001

Animals were exposed to 3.75 or 5 mg/kg/day/4 days, and sperm cells were collected and analyzed at 1-, 28- or 45-dpt. dpt = days post-treatment.

Table 4

MET exposure did not induce lipoperoxidation or alter mitochondria in spermatozoa collected at 1-, 28- or 45-dpt.

Group	MDA ($\mu\text{M}/5 \times 10^5$ spermatozoa)	MMP (red/green fluorescence)
MET (mg/kg/day/4 days) at 1-dpt		
Control	9.17 \pm 4.03	100 \pm 1.5
5	11.65 \pm 3.87	94.95 \pm 0.54
MET (mg/kg/day/4 days) at 28-dpt		
Control	3.65 \pm 1.07	93.28 \pm 9.64
5	4.61 \pm 1.97	90.87 \pm 10.17
MET (mg/kg/day/4 days) at 45-dpt		
Control	3.27 \pm 0.09	99.99 \pm 32.17
5	5.54 \pm 1.60	99.84 \pm 27.35

Data are expressed as the mean \pm SD (n = 8 controls; n = 12 treated animals per dose) from two independent experiments. MDA = malondialdehyde; MMP = mitochondrial membrane potential; and ND = not determined. No significant differences (p > 0.05) were observed compared to control mice according to the non-parametric Mann–Whitney test.

in phosphorylation was observed for serine residues (67%) (Fig. 5a) in bands from 12 to 73 kDa (Fig. 6c), and the increment for tyrosine residues was 21% (Fig. 5b) in bands between 42 and 68 kDa (Fig. 6f). Interestingly, some bands decreased in intensity at serine or tyrosine residues in MET-treated groups, such as those of approximately 110 kDa or higher in cells collected at 1-dpt.

Discussion

Anticholinesterase pesticides are widely used in Mexico, and currently, MET is one of the most used insecticides in some areas of the country (Blanco-Muñoz et al., 2010; Pérez-Herrera et al., 2008; Rojas-García et al., 2011), though its use has been restricted (NRA, 2002). This represents a public health problem because agricultural workers are frequently exposed to high doses of pesticides during spraying seasons, as illustrated by the thousands of intoxication episodes registered yearly (AMIFAC, 2013). MET was studied for its potential to both alter embryo development after paternal exposure in experimental animals (Burrue et al., 2000; Farag et al., 2012) and induce genetic changes in somatic cells (Amer and Sayed, 1987).

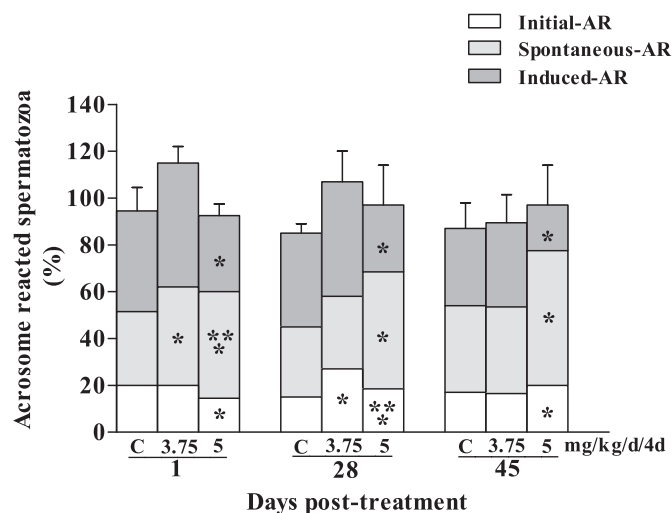


Fig. 2. MET exposure effects on the acrosome reaction. Spermatozoa were collected at 1-, 28- or 45-dpt and the percentage of spermatozoa without acrosome or undergoing the spontaneous- or induced-AR in capacitation conditions were obtained. Data represent the median \pm SE (n = 8 control mice and n = 12 treated mice per dose). *Significant difference compared to control mice (p < 0.001) according to the Wilcoxon U test. **Significant difference compared to the 3.75 mg/kg/day group (p < 0.05) according to the Kruskal–Wallis test. C = control.

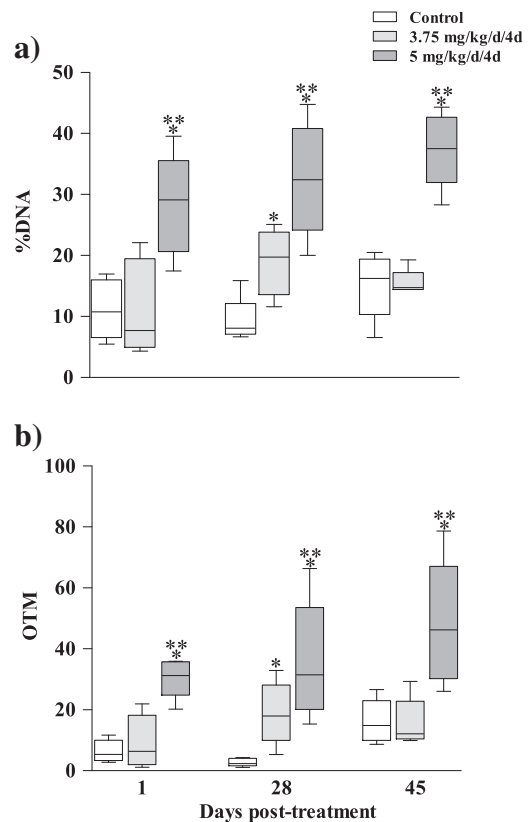


Fig. 3. MET effects on sperm DNA. Spermatozoa were collected at 1-, 28- or 45-dpt and analyzed by the comet assay. a) %DNA and b) OTM parameters. Data represent the median, 75th percentile and maximum and minimum (n = 8 controls and n = 12 treated mice per dose). *Significant difference compared to control mice (p < 0.01) according to the Wilcoxon U test. **Significant difference compared to the 3.75 mg/kg/day group (p < 0.05) according to the Kruskal–Wallis test.

Therefore, we evaluated the acute effects of MET on male reproduction, particularly sperm function, exploring the sensitivity of three stages of spermatogenesis, namely mitosis, meiosis and epididymal maturation. We evaluated the effects of high MET doses that may be related to intoxications; few studies have reported MET concentrations in humans, but did not present data of MET levels in intoxication episodes (Montesano et al., 2007). We showed that acute MET exposure impaired sperm function affecting cells at the three stages of maturation, in which the testicular stages are more sensitive. Specifically, cells first exposed in testes

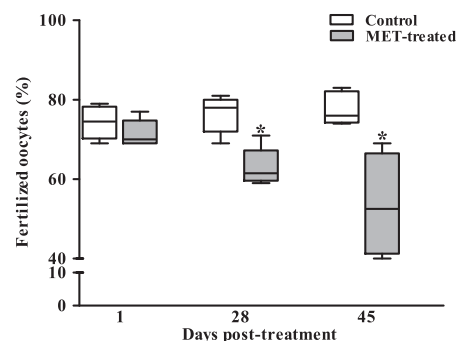


Fig. 4. Effects of MET exposure on sperm fertilization capacity. Spermatozoa from mice exposed to 5 mg/kg/day/4 days were collected at 1-, 28- or 45-dpt and tested for their in vitro ability to fertilize zona pellucida-intact oocytes. Data represent the median, 75th percentile and maximum and minimum (n = 8 controls and n = 12 treated mice per dose). A total of 100 oocytes were examined in each group. *Significant difference compared to control mice (p < 0.05) according to the Wilcoxon U test.

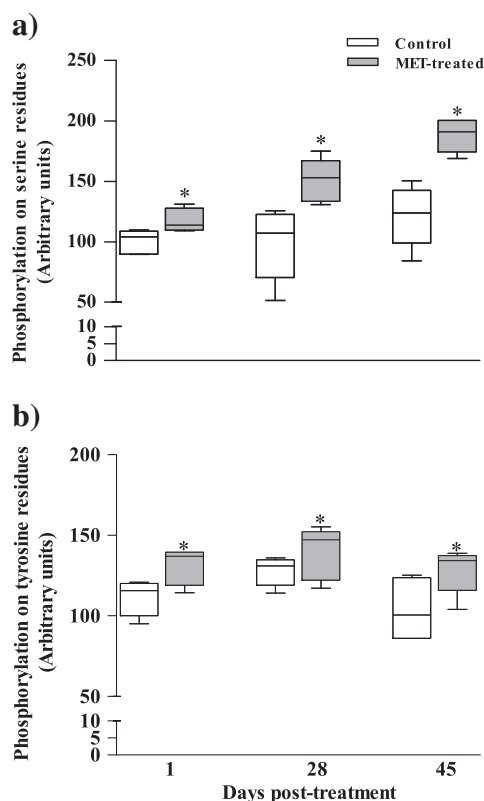


Fig. 5. Protein phosphorylation in sperm homogenates after MET exposure. Sperm cells from mice exposed to 5 mg/kg/day/4 days were collected at all time points, homogenized and evaluated for a) phosphorylation of serine residues using an anti-phosphoserine antibody and b) tyrosine phosphorylation using an anti-phosphotyrosine antibody. Data represent the median, 75th percentile and range ($n = 8$ controls and $n = 12$ treated mice per dose). Comparisons between treated and control groups ($*p < 0.05$) were examined according to the Wilcoxon U test.

and collected at 28- and 45-dpt were vulnerable to MET toxicity; sperm quality was reduced, acrosome formation and function were altered, fertilization ability was diminished and sperm DNA damage was observed. Other pesticides have also shown effects on pachytene spermatocytes (28-dpt) such as Me-Pa (Piña-Guzmán et al., 2009) and the fungicide carbendazim (Kadalmani et al., 2002). Most relevant exposure pathways to pesticide in occupational settings are inhalation, ingestion and cutaneous contact, however, the administration of chemicals by ip injection is an approach to study their mechanisms of toxicity. Thus, our conclusion that several stages of spermatogenesis are affected by acute high exposures to pesticides indicates that sperm cells remain affected weeks after the exposure occurs, with consequences in reproduction. These data may be relevant for being considered in the risk assessment of pesticide exposures in humans.

Because the integrity of the acrosome is essential for fertilization, premature or delayed AR may prevent sperm penetration to the zona pellucida (Bedford and Cross, 1999). In this study, we evaluated initial-, spontaneous- and induced-AR (by GABA stimulation), and found that spermatozoa collected at 1-, 28- and 45-dpt had a significantly decreased ability to undergo induced-AR and an increased percentage of spermatozoa displayed spontaneous-AR at the highest dose. The premature AR observed at all time points can be linked to the reduced number of spermatozoa undergoing AR in response to GABA. This finding suggests that acrosome proteins or membrane components were altered and the acrosome function was therefore altered. The acrosome has been reported to be a target of the toxicity of pesticides such as Me-Pa after single doses in mice (Piña-Guzmán et al., 2009), beta-cypermethrin after repeated doses in mice (Wang

et al., 2009), and endosulfan and chlordane in human sperm cells treated in vitro (Turner et al., 1997).

AR alterations observed at 28-dpt suggest that MET exposure is able to induce a structural disruption in the Golgi apparatus, which is the precursor organelle of the acrosome in spermatocytes (Anakwe and Gerton, 1990), whereas the effect at 45-dpt could be the result of alterations (phosphorylation or alkylation) on proteins or genes that are involved in acrosome formation or function, possibly by epigenetic mechanisms (discussed later). On the other hand, alterations observed in spermatozoa collected at 1-dpt (the epididymal maturation stage) could be due to acrosome membrane damage during the epididymal transit where changes in membrane components occur (Jones, 1998), although the effect observed at this stage was most likely not strong enough because the fertilization rate was not affected.

Fertilization ability is an essential endpoint for evaluating male reproductive function (Berger et al., 2000); therefore, we evaluated the in vitro fertilization rate after exposure to MET. We observed a diminished fertilization potential in spermatozoa exposed during mitosis (collected at 45-dpt), a smaller decrease in spermatozoa exposed during meiosis (collected at 28-dpt), and no effect on the fertilization ability in spermatozoa exposed during the epididymal transit (collected at 1-dpt). The effects observed in cells that were at meiosis and mitosis at first exposure to MET could be a consequence of alterations in the acrosome due to disturbances during the acrosome formation as discussed above, which will result in fewer spermatozoa that are able to undergo AR. The phosphorylation of sperm proteins by MET exposure reported here may also contribute to the decreased fertilization rate if altered proteins are involved in any of the processes required for proper fertilization (i.e., capacitation, zona binding and/or membrane fusion). We cannot discard that sperm DNA alterations may also be involved in the decreased fertilization observed in this study because DNA integrity and proper chromatin condensation are key factors in the fertilization process (Sun et al., 1997). Duran et al. (2002) showed that samples with $> 12\%$ of sperm DNA fragmentation (TUNEL) resulted in failed pregnancies.

The toxicity of OP exposure may be due to three main mechanisms: protein phosphorylation, protein or DNA oxidation and DNA alkylation (i.e., the formation of alkylated adducts). Many studies on adverse effects of OP pesticide exposures in sperm cells have proposed that the oxidative stress resulting from metabolism via the cytochrome P450 (CYP) pathway provides a mechanism of action (Piña-Guzmán et al., 2006; Sarabia et al., 2009; Wang et al., 2009); however, in the case of MET, no oxidative effect was observed, possibly because MET does not require oxidative activation by the CYP pathway because it is chemically an oxon. This finding suggests that MET toxicity in male germinal cells occur through other mechanisms, although additional endpoints to test oxidative damage are needed to make this conclusion. One of the most well-known mechanisms of OP toxicity, mainly neurotoxicity, is protein phosphorylation (Tacal and Lockridge, 2010). We recently reported that single doses of the OP insecticide diazinon in mice phosphorylated nuclear sperm proteins (protamines), thus altering the sperm chromatin structure (Piña-Guzmán et al., 2005). Therefore, we conducted experiments to evaluate the ability of MET to phosphorylate proteins in sperm cells. The immunodetection analyses showed that both serine and tyrosine residues are phosphorylated in proteins from spermatozoa collected at all time points, although the testicular stages (28- and 45-dpt) seemed to be more severely affected, and only tyrosine phosphorylation was dependent on the time of collection. Therefore, these alterations in proteins may explain some of the effects observed in the sperm function of mice exposed to MET. Further studies are in progress to identify the target proteins and their role in mediating MET toxicity on sperm function. Interestingly, we noticed the decrease in some bands of high molecular weights (≥ 110 kDa), particularly at 1-dpt, that deserves a deeper evaluation.

An interesting result observed in this study was the ability of MET to cause DNA damage in sperm cells at the three stages of spermatogenesis,

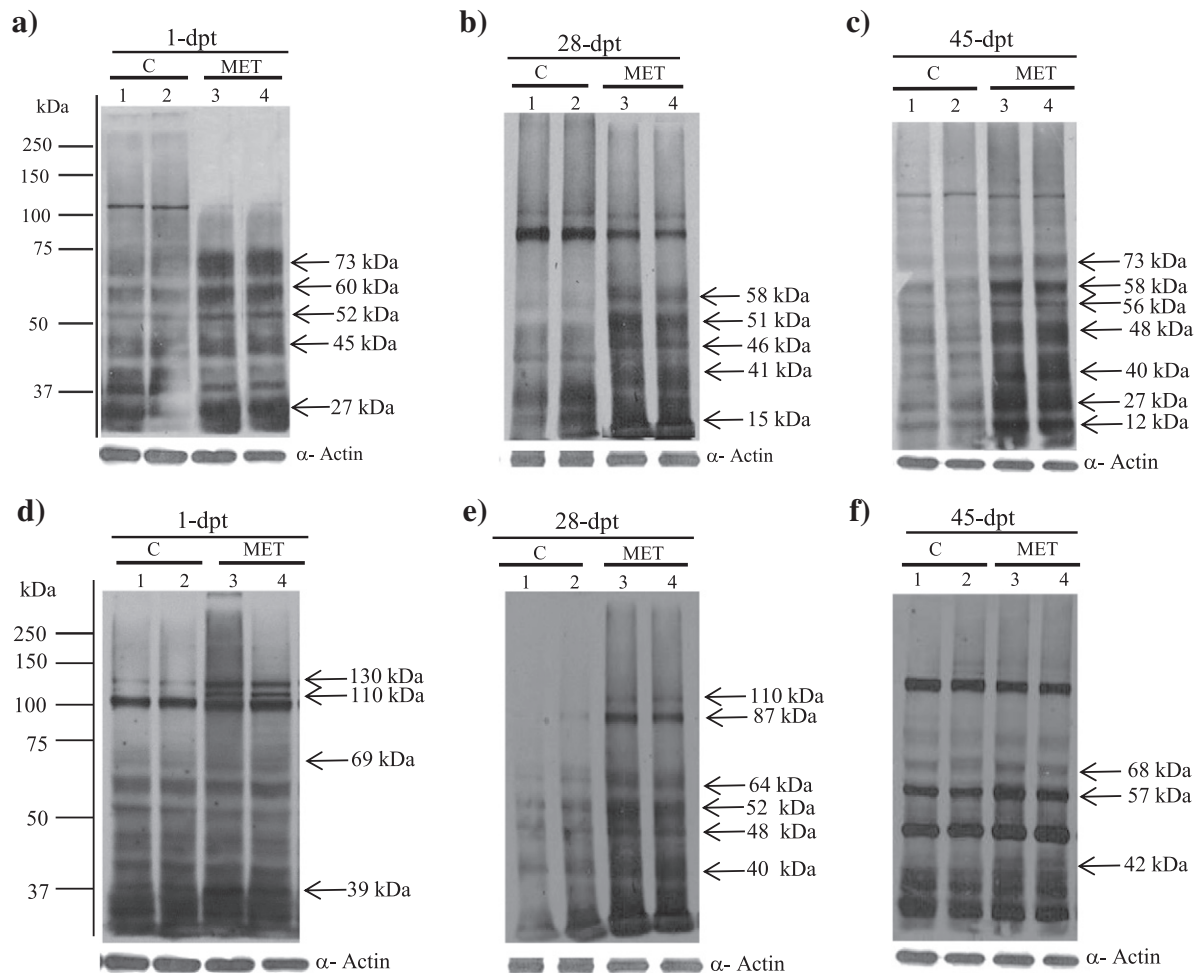


Fig. 6. Immunodetection of protein phosphorylation in sperm homogenates after MET exposure. Spermatozoa from mice exposed to 5 mg/kg/day/4 days and collected at 1-, 28- and 45-dpt were homogenized, separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with a) anti-phosphoserine antibodies and b) anti-phosphotyrosine antibodies. Black arrows indicate bands showing a higher fluorescence signal compared to control sperm cell homogenates.

alterations that were not repaired because the DNA damage was observed in mature cells collected at 1-, 28- and 45-dpt. This damage may be related to both mechanisms that have already been discussed: oxidation of proteins or DNA and phosphorylation of proteins, which in turn may disrupt DNA function and expression. As mentioned above, diazinon phosphorylates nuclear sperm protamines, thus altering the sperm chromatin structure and DNA integrity in mice (Piña-Guzmán et al., 2005). We also showed that Me-Pa caused the formation of the oxidized adduct 8-OHdG in sperm cells after Me-Pa exposure in mice (Monroy-Peréz et al., 2012).

A third mechanism of DNA damage caused by MET exposure can be the alkylation of nucleic acids. The genotoxic potential of OPs on somatic cells has been extensively studied (Eto, 1974). Zayed and Mahdi (1987) showed DNA methylation in somatic cells exposed to MET, reporting the formation of [7-¹⁴C] methylguanine in mouse liver cells after 6 h of exposure to [OCH₃-¹⁴C]MET. However, the information on genotoxic effects on germinal cells is limited. Padungtod et al. (1999) reported a high frequency of sperm aneuploidy (X,Y and 18) in workers occupationally exposed to ethyl-parathion and MET, and Recio et al. (2001) observed similar results in agricultural workers exposed to a mixture of OPs, including MET. Meecker et al. (2004) suggested that environmental exposure to chlorpyrifos may be associated with increased DNA damage in human sperm, and studies from our group have shown that sperm chromatin structure and DNA integrity are targets of OP exposure in agricultural workers exposed to mixtures of OPs (Pérez-Herrera et al., 2008; Sánchez-Peña et al., 2004), and in mice exposed to Me-Pa

(Piña-Guzmán et al., 2005) or diazinon (Piña-Guzmán et al., 2005). Alkylating agents can attack not only DNA but also proteins; it was reported that the alkylation of sperm nuclear protamines causes a significant stress on chromatin structure leading to DNA damage (single- or double-strand DNA breaks) (Evenson et al., 1993; Segal, 1991).

Chemical alterations caused by OPs, in particular MET, such as protein phosphorylation and/or DNA or protein alkylation could be considered as epigenetic mechanisms of toxicity. Currently, epigenetic modifications, including methylation loss or gain, which in turn depend on histone or protamine configuration can be involved in fertility problems. The importance of epigenetic processes in the paternal DNA genome for male fertility and for embryonic development has been reviewed (Boissonnas et al., 2013). Furthermore, increasing evidence suggests that genetic and environmental factors can have negative effects on epigenetic processes controlling implantation, placentation and fetal growth (Dada et al., 2012). Therefore, the DNA damage observed after MET exposure may contribute to effects observed in embryo development by paternal exposure to MET. This includes the impairment of embryonic cell progression and the increase in the number of degenerated embryos observed by Burrueal et al. (2000), or the decrease in the male fertility index shown by a decrease in pup survival and the increase of uterine resorptions (Farag et al., 2012).

Our results showed that MET acute exposure in mice causes several effects, including low sperm quality, acrosome alterations, protein phosphorylation and DNA damage, that together led to a decreased

fertilization ability of sperm cells, where the testicular stages of spermatogenesis are the most sensitive. These findings gain importance when considering that even after ending the spraying season, the effects of MET persist in sperm cells during one spermatogenic cycle. Additionally, the DNA damage caused by MET may have consequences for progeny, and further research is needed to determine the mechanism of MET damage, whether it is through a direct alteration on DNA (methylation) and/or indirectly by chemical modifications of nuclear proteins (phosphorylation/alkylation).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2014.06.017>.

Statement of conflict of interest

All authors declare that there are no conflicts of interest and that they have approved the manuscript.

Acknowledgments

The authors want to thank Ricardo Gaxiola Centeno and Rafael Leyva Muñoz for their technical assistance with the animals, Víctor H. Rosales García for his assistance with flow cytometry, Alberto Hernández Alcántara for his help with the comet assay, and Dr. Leticia Hernández-Cadena for her critical discussion of statistical analyses. Also we want to acknowledge the generosity of Dr. Manuel Hernández-Hernández from the Department of Cell Biology (CINVESTAV-IPN) for the gift of the anti-actin primary antibody. This study was supported by CONACYT-Mexico (Grant #58213 given to BQV). MUA was a recipient of a scholarship from CONACYT-Mexico.

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