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

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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.

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 methyl-parathion (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-^{14}C] methylguanine in mouse liver cells after exposure to [OCH$_3$-^{14}C]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 Amershan Life Science (Amersham, UK), and JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazoyl carbocyanine iodide) and carbonyl cyanide m-chlorophenylhydrazone (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 Pellco 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 LD_{50} reported by Burruel 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 DL_{50}. 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 μl 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