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

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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, possibly via oxidative damage. This study investigated the stages of spermatogenesis susceptible to be targeted by Me-Pa exposure that impact on spermatozoa function and their 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. Spermatozoa were examined for DNA damage 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 fluorochrome, and fertilization ability by an *in vitro* assay and *in vivo* mating. Alterations on DNA integrity (%DFI and NT-positive cells) in spermatozoa collected at 7 and 28 dpt, and decreases in sperm quality and induced-AR were observed; reduced MMP and LPO were observed at 7 dpt only. Negative correlations between LPO and sperm alterations were found. Altered sperm functional parameters evaluated either *in vitro* or *in vivo* were associated with reduced fertilization rates at both times. 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 exposure in male germ cells.

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 (Jokanovic, 2001), and inhibition of acetylcholinesterase (AChE) followed by accumulation of acetylcholine (Rubin 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 oxidative effects of OP compounds on the mammalian reproductive system have been reported. 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

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.

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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 have not yet been determined. In the current study, we investigated the effects of male exposure to Me-Pa on the function and fertilizing capacity of male germ cells as well as 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 corresponds to 1/3 of the LD_{50} in mice (Goyer and Cheymol, 1967), was the highest non-lethal dose 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 AR assays; six control and six treated animals).

Sperm quality evaluation. Sperm evaluation included cell concentration, viability, progressive motility, and normal morphology according to World Health Organization guidelines (WHO, 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 *D*NA *F*ragmentation *I*ndex (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 in 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 dUTP (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,