Reproductive Toxicology 25 (2008) 455–460

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Sperm chromatin alteration and DNA damage by methyl-parathion, chlorpyrifos and diazinon and their oxon metabolites in human spermatozoa

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ARTICLE INFO

Article history:
Received 30 October 2007
Received in revised form 23 April 2008
Accepted 15 May 2008
Available online 21 May 2008

Keywords:
Oxons toxicity
Methyl-parathion
Chlorpyrifos
Diazinon
Sperm chromatin
Sperm DNA

ABSTRACT

Extensive use of organophosphorous pesticides (OP) by young men represents a public health problem. Toxicity of OP mainly results in neurotoxicity due to their oxygen analogues (oxons), formed during the OP oxidative activation. OP alter semen quality and sperm chromatin and DNA at different stages of spermatogenesis. Oxons are more toxic than the parent compounds; however, their toxicity to spermatogenic cells has not been reported. We evaluated sperm DNA damage by several OP compounds and their oxons in human spermatozoa from healthy volunteers incubated with 50–750 μM of methyl-parathion (MePA), methyl-paraoxon (MePO), chlorpyrifos (CPF), chlorpyrifos-oxon (CPO), diazinon (DZN) or diazoxon (DZO). All concentrations were not cytotoxic (evaluated by eosin-Y exclusion), except 750 μM MePO. Oxons were 15% to 10 times more toxic to sperm DNA (evaluated by the SCSA parameter, %DFI) than their corresponding parent compounds, at the following order: MePO > CPO = MePA > CPF > DZO > DZN, suggesting that oxon metabolites participate in OP sperm genotoxicity.

1. Introduction

Organophosphorous pesticides (OP) are among the most worldwide-used agrochemicals; are mainly known by their neurotoxic effects due to the inhibition of acetylcholinesterase (AChE) by their oxygen analogues, which are the active metabolites generated during OP metabolism [1]. However, other toxic effects of OP, such as those in the male reproductive system, have attracted attention. Studies in men and animals have demonstrated that OP alter male reproductive function, particularly semen quality and hormone balance [2–5].

Among the most used and toxic OP is methyl-parathion (MePA; *O*,*O*-dimethyl-*O*-4-*p*-nitrophenyl phosphorothioate), an insecticide that has been shown to induce genotoxic effects. It has been reported that MePA induces sister chromatid exchange in human lymphocytes and it was demonstrated its ability to interact directly with double-stranded DNA disturbing its stability and conformation [6,7]. The phosphorotioate diazinon (DZN; *O*,*O*-diethyl-*O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester) is another OP insecticide that has been described as a mutagenic agent in somatic cells using in vivo and in vitro systems [8–10]; while chlorpyrifos (CPF; *O*,*O*-diethyl-*O*-[3,5,6-trichloro-2-pyridinyl] phosphorotionate) is a pesticide with an increased use in the last decade, which has been shown to cause dose-related DNA damage in mice lymphocytes [11].

Despite the reports about the genotoxic potential of several OP in somatic cells [12], the information in germinal cells is limited. Sperm DNA integrity is protected by its highly compacted structure, however, it has been shown to be target by environmental agents like OP. Padungtod et al. [13] and Recio et al. [14] reported an increased frequency of sperm aneuploidy in men occupationally exposed to OP mixtures (ethyl-parathion (EtPA), MePA, methamidophos and diazinon (DZN), among them), and Meeker et al. [15] found that environmental exposure to CPF can be associated with increased sperm DNA fragmentation. In vitro experiments have demonstrated that EtPA and its metabolite paraoxon affect sperm chromatin condensation [16], and previous work from our group demonstrated that sperm chromatin integrity is altered in

Abbreviations: MePA, methyl-parathion; MePO, methyl-paraoxon; EtPA, ethyl-parathion; DZN, diazinon; DZO, diazoxon; CPF, chlorpyrifos; CFO, chlorpyrifos-oxon; DFI, DNA fragmentation index.

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doi:10.1016/j.reprotox.2008.05.055

agricultural workers exposed to OP mixtures, including MePA, DZN and methamidophos [5], and in mice acutely exposed to DZN or MePA [4,17].

The genetic damage on sperm cells depends of the spermatogenic stages being exposed, among other variables, and the unique sensitivity of sperm chromatin from mature spermatozoa to be altered by the interaction of reactive chemicals with nuclear sperm proteins has been demonstrated from our previous studies with lead [18] and OP [4,17], and from a study with alkylating agents [19]; this sensitivity is also related to the high content of spermatozoa of unsaturated fatty acids-enriched membranes and their lack of antioxidant and DNA-repair systems [20].

It is known that defects on the genomic integrity of male gametes are indicative of infertility [21,22], defects in embryo development [23,24], and linked to an increased incidence of cancers in the offspring [25]. In this regard, Burruel et al. [26] reported that acute paternal exposure to methamidophos showed an increase number of degenerated embryos, and Farag et al. [27] showed that an oral dimethoate administration in male mice decreased the number of implantation sites and live fetuses after mating with un-treated females; these data suggest that OP have the ability to produce transmissible adverse effects.

Among OP, MePA, CPF and DZN, are commonly used insecticides that require bioactivation through an oxidative desulfuration to form their oxygen analogues (the oxons), which are the metabolites with the greatest neurotoxic action (inhibition of AChE) [28]. OP oxidative desulfuration has been reported in several tissues, including testis [29], and several CYP isoforms have been reported to bioactivate OP in human hepatic microsomes [30]. Results from our group have shown the presence of some CYP isoforms involved in MePA activation in mice testis and epididymis–vas deferens (manuscript in preparation), suggesting that the male reproductive tract has the ability to bioactivate OP in situ, thereby generating the toxic highly reactive intermediates such as the oxons and/or reactive oxygen species (ROS).

It has been extensively reported that the oxygen analogues (oxons) of OP are more potent neurotoxins than their parent compounds [28]; however, scared information is available about the oxons toxicity and genotoxic potential to spermatogenic cells. Thus, to determine the genotoxicity of OP metabolites to male germinal cells, sperm DNA damage and chromatin structure were evaluated in mature spermatozoa exposed to several OP parent compounds and their corresponding oxons.

2. Methods

2.1. Chemicals

Chemical grade MePA, methyl-paraoxon (MePO), DZN, diazoxon (DZO), CPF and chlorpyrifos-oxon (CPO) were purchased from Chem. Service (West Chester, PA). DMSO and eosine Y were provided by Sigma (St Louis, MO) and acridine orange (AO) from Amersham (Amersham, UK). All other chemicals were of reagent grade.

2.2. Sperm samples

Semen samples were obtained from three healthy volunteers, who proved having normal parameters of semen quality according to WHO [31], and no sperm DNA damage (DFI < 15%; DFI = DNA fragmentation index) [32]. Semen samples (three samples per individual) were collected in sterile wide-mouth containers, and screened for quality within 1 h of collection.

2.3. Pesticide incubations

Semen samples from three donors were assigned in aliquots of 5×10^6 spermatozoa (with at least 70% progressive motility) and incubated with OP parent compounds or their metabolites at concentrations of 50, 300, 500 and 750 μM in DMSO (0.15%) and BWW capacitating medium containing bovine serum albumin (3.5 mg/ml). Each concentration was replicated six times. Incubations were done in 1 ml final volume for 1 h at 37 °C in an atmosphere 5% CO_2 and 95% O_2. The control samples were incubated in BWW medium containing DMSO free from the tested OP compounds. Separate experiments were carried out with semen samples from each donor collected at different time intervals.

2.4. Cytotoxicity assay

Viability was evaluated according to WHO guidelines [31], using 0.5% eosin Y. The dye exclusion was evaluated in 100 spermatozoa in an Olympus BX40 microscope and expressed as percentage of viable cells.

2.5. Sperm chromatin structure assay (SCSA)

Sperm DNA integrity was assessed by the SCSA described by Evenson and Melamed [33]. The 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 intercalates into native double-stranded DNA, and emits a red fluorescence (>630 nm) when intercalates into denatured single-stranded DNA. The extent of DNA denaturation is quantified by the DFI, which is the ratio of red to red + green fluorescence, and the extent of DNA denaturation for each sperm is expressed as the proportion 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).

2.6. Statistical analyses

Results are expressed as mean ± S.D. of three independent samples from each volunteer replicated six times. Treated and control groups were compared by analysis of variance with Bonferroni post-test. Dose–response effects were evaluated by using linear regression analysis, as well as to estimate the concentration of different OP tested compounds to elicit a DNA damage of DFI ≥ 30%. Normality of the SCSA parameters was assessed, and appropriate transformations were performed before linear regression: %DFI from MePA, CPF, CPO, DZN and DZO as well as mean DFI from MePA were normally distributed, while mean DFI from CPF, DZN and DZO were cubic-transformed and %DFI and mean DFI from MePO were transformed to square-root. In addition, the linear regression equation: $X = (y - \beta_0)/\beta_1$ was used to estimate the concentration of different OP tested compounds to elicit sperm DNA damage, considered as DFI ≥ 30%, where X represents the concentration of the different OP compounds that may elicit a DFI ≥ 30%; y is 0.30 which represents the DFI value of 30%; β_0 and β_1 are values derived from the linear regression analysis, β_0 is the predict constant value whereas β_1 is the regression coefficient. The STATA program Version 8.0 (Stata Corp., College Station, TX) was used for all statistical calculations. We considered p-values <0.05 to be statistically significant.

3. Results

To address if the oxon metabolites of OP were genotoxic to mature spermatozoa, we incubated human ejaculated spermatozoa with different concentrations of three OP insecticides and their corresponding oxon metabolites. Results presented in Table 1 show that the concentrations (50–750 μM) of the six different tested compounds were not cytotoxic to mature sperm cells, since the percentage of viable cells at all concentrations was higher than 80%, except at 750 μM MePO that shows a 76% of viability.

Regarding the effects on the sperm DNA integrity evaluated by the SCSA parameter, %DFI, that represents the percentage of cells showing DNA damage, results are presented in Figs. 1–3. The variation among individuals and among samples from each volunteer was small (<10%). All OP compounds caused DNA damage with respect to the controls, however the degree varied. MePA and MePO showed significant effects on %DFI at 300 μM and above, although the oxon metabolite was more toxic (Fig. 1A). Increases in %DFI after MePA incubation were of 28, 40 and 70% at 300, 500 and 750 μM, respectively, compared with the control spermatozoa, while the increases after MePO incubation were from fourfold at 300 μM to eightfold at 750 μM. On the other hand, Fig. 2 depicts the effects of DZN and DZO exposure on %DFI. A significant increase on %DFI after DZN incubation was observed only at 500 and 750 μM with values of 13 and 33%, respectively, compared with the controls, and the increase on %DFI after DZO incubation was observed at doses of 300 μM and above, with values from 31 to 65% the controls (Fig. 2A). Finally, the incubation of spermatozoa with CPF and CPO resulted

Table 1
Spermatozoa viability after incubation with different OP compounds

Concentration (μM)	Viability (%)					
	Methyl-parathion	Methyl-paraoxon	Chlorpyrifos	Chlorpyrifos-oxon	Diazinon	Diazoxon
Control	96.3	96.7	97.3	94.81	97.9	93.2
50	98.2	96.0	95.9	93.4	97.7	91.5
300	95.1	90.1	93.1	89.4	93.0	90.5
500	96.3	83.8*	86.2	83.0*	91.8	84.3
750	81.4*	75.8*	81.7*	80.3*	86.9	81.1*

Data are presented as mean from three independent experiments from each donor done by sextupling.
* Statistically significant with respect to the control cells, $p < 0.05$.

in less-pronounced effects on the %DFI parameter. The increase on %DFI after the incubation with the parent compound was observed only at 500 and 750 μM, with significant increases of 33 and 45% the control value, respectively, and from 7 to 47% at 300 μM and above after the incubation with its oxon metabolite (Fig. 3A).

The relationships between the tested compounds and the DNA damage (%DFI parameter) are presented in Table 2. All the OP parent compounds and their oxon metabolites showed dose–response effects with correlation values from 0.7852 for DZO to 0.9731 for MePO.

Considering the SCSA parameter mean DFI, that represents the sperm chromatin condensation, it was also affected by the incubation with the OP parent compounds and their oxon metabolites. Increases from 6 to 14% the control value were observed after MePA incubation at doses of 300 μM and above (Fig. 1B), and from 48 to 74% after MePO incubation. While, significant albeit small increases of 4 and 6% were observed after DZN incubation at doses of 500 and 750 μM, respectively, and similar increases (about 5%) were observed after DZO incubation at 300 μM and above (Fig. 2B). Similarly, significant increases of 6 and 10% on mean DFI were observed at 500 and 750 μM of CPF incubation and from 5 to 10% at doses of 300 μM and above of CPO.

In order to estimate the concentration of the tested OP parent compounds and their corresponding oxidative metabolites at which fertility is compromised (DFI ≥ 30%) [32], a linear regression analysis was applied, generating the best estimates shown in Table 3. This parameter (%DFI) has been widely used in epidemiological studies in the andrology field to estimate the reproductive potential as well as in toxicology for the identification of potential reproductive hazards [32]. Results from this analysis showed that the genotoxicity order was: MePO > MePA = CPO > CPF > DZO > DZN; therefore, these values indicate that MePO, the oxon metabolite

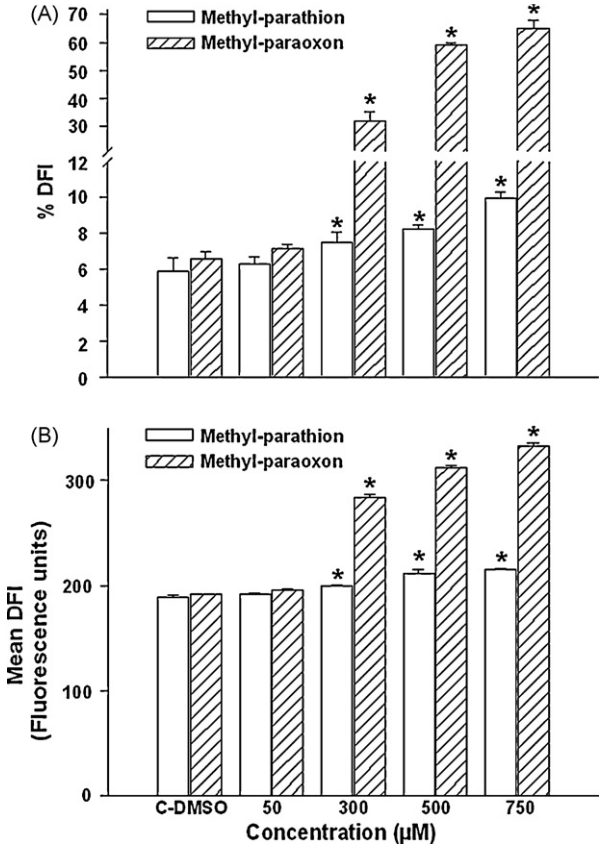

Fig. 1. Effects of methyl-parathion and methyl-paraoxon on the sperm chromatin structure. (A) %DFI and (B) mean DFI. Bars represent mean ± S.D. ($n = 3$ separate experiments made by sextupling from each donor). *$p < 0.05$ vs. the control group, according to the analysis of variance (ANOVA) and the Bonferroni multiple comparison test. C-DMSO = control group added with DMSO.

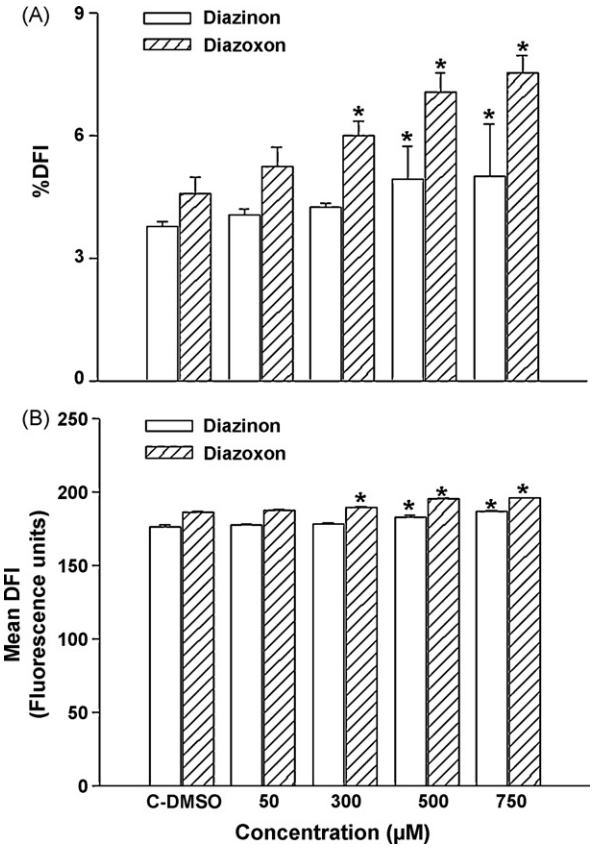

Fig. 2. Effects of chlorpyrifos and chlorpyrifos-oxon on the sperm chromatin structure. (A) %DFI and (B) mean DFI. Bars represent mean ± S.D. ($n = 3$ separate experiments made by sextupling from each donor). *$p < 0.05$ vs. the control group, according to the analysis of variance (ANOVA) and the Bonferroni multiple comparison test. C-DMSO = control group added with DMSO.

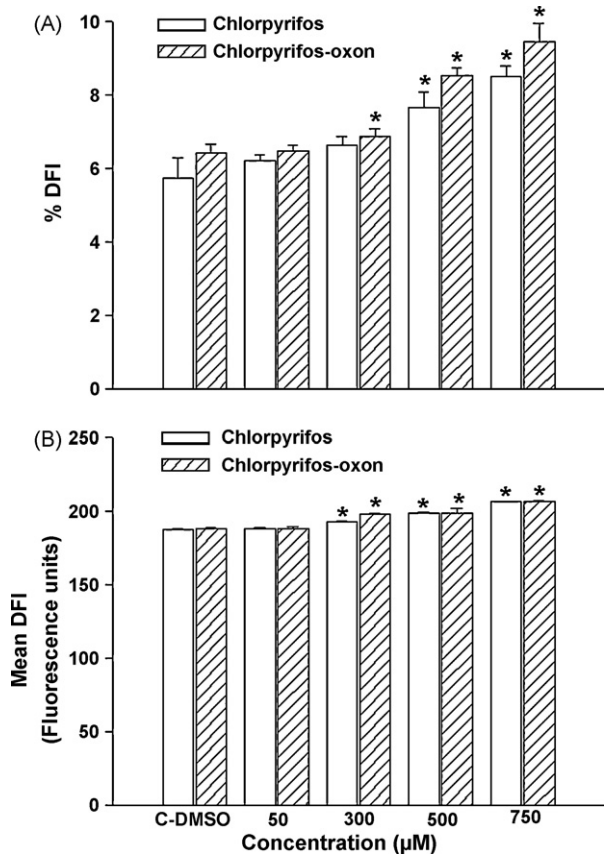

Fig. 3. Effects of diazinon and diazoxon on sperm chromatin structure. (A) %DFI and (B) mean DFI. Bars represent mean ± S.D. ($n = 3$ separate experiments made by sextupling from each donor). *$p < 0.05$ vs. the control group, according to the analysis of variance (ANOVA) and the Bonferroni multiple comparison test. C-DMSO = control group added with DMSO.

Table 2
Relationships between different OP compounds and sperm DNA damage

Compound	%DFI	
	β	r^2
Methyl-parathion	0.0071	0.8043*
Methyl-paraoxon	0.0617	0.9731*
Chlorpyrifos	0.0061	0.8889*
Chlorpyrifos-oxon	0.0068	0.8992*
Diazinon	0.0025	0.8215*
Diazoxon	0.0040	0.7852*

A simple linear regression analysis was done. β represents the regression coefficient; r^2 represents the reliability of the linear relationship between %DFI and OP concentrations.
* $p < 0.001$.

Table 3
Estimation of OP concentrations that may cause sperm DNA damage (DFI) ≥30%

Compound	Concentration[a] (mM)
Methyl-paraoxon	0.332
Methyl-parathion	3.435
Chlorpyrifos-oxon	3.549
Chlorpyrifos	4.302
Diazinon	6.238
Diazoxon	10.251

[a] Concentration of OP compounds that cause a DFI ≥30% (value that compromises fertility in humans) [32] according to a simple linear regression analysis.

of MePA, is the most toxic compound to mature spermatozoa (0.332 mM to cause 30% of DFI) and the parent compound DZN is the least toxic (10.25 mM to cause 30% of DFI).

4. Discussion

Genotoxicity of OP has been contradictory reported in somatic cells [12], but limited information is available on male germinal cells. Sperm DNA is normally resistant to aggressors because of its highly compacted structure, but it has been proposed as a target of environmental agents like OP. We have previously reported that OP exposure alters sperm chromatin but the mechanisms have not been elucidated. As a first approach to evaluate the participation of the oxygen analogues of OP on the toxicity to male germinal cells, we conducted this study in mature spermatozoa (one of the sensitive cell types of the spermatogenic lineage), and showed the genotoxic potential of the oxon metabolites of several OP, as well as of the parent compounds, at concentrations that were not cytotoxic. Our results suggest that the oxygen analogues of MePA, DZN and CPF have the ability to damage mature sperm chromatin, particularly the DNA, being more toxic than their corresponding parent compounds and that OP metabolism may be involved in this genotoxicity.

Epidemiological studies have reported sperm chromatin/DNA alterations in men exposed to several OP, taking as exposure indicator the urinary excretion of OP metabolites (dialkylphosphates) in urine [5,13–15], but there is not available information about the levels of OP compounds or their metabolites in semen. Unpublished results [34] reported low concentrations (μM range) of OP parent compounds in semen from urban pest control sprayers; however, agricultural workers are exposed to higher concentrations of OP pesticides in the spraying season that may reach high concentrations in the reproductive tract. Furthermore, our results from experimental models have suggested that epididymal spermatozoa are directly exposed to high concentrations of OP metabolites produced in situ in the reproductive tract that may not necessarily be monitored in semen [17]. In addition, concentrations of OP compounds tested in this study were according to other in vitro studies in which concentrations in the range of 36 to >500 μM of MePA, MePO or dimethoate showed no cytotoxic effects in human lymphocytes but a significant increase in DNA damage [35,36]; and Contreras et al. [16] observed no difference in the effects of EtPA and its oxon analogue on sperm chromatin decondensation after in vitro incubations to similar concentrations (0.1–0.8 mM) than those tested in this study. In vitro methods have made significant contributions to our understanding of mechanisms of toxicity; therefore, the genotoxic effects observed here in epididymal spermatozoa after the incubation with OP oxon metabolites strongly suggest the importance of exploring this effect and its mechanism of action after in vivo exposures.

Sperm chromatin/DNA integrity is essential for the proper transmission of paternal genetic information; therefore, sperm chromatin structure is important for sperm fertilizing ability. The sperm chromatin structure assay (SCSA) was developed by Evenson and Melamed [33] and has been extensively used in the last decades as a useful biomarker of human fertility potential and human toxin exposure. Recently, Evenson and Wixon [37] concluded from their meta-analysis, that existent literature continue to support that semen samples with approximately >30% DFI are associated with decreased pregnancy outcomes and in vitro fertilizations, as well as with increased risk for early spontaneous miscarriages. In this regard, MePO was the only tested OP compound that may cause a potential risk on spermatozoa ability to fertilize.

Previous studies from our group and others have demonstrated that alterations in sperm chromatin structure depend, among other

factors, on the spermatogenic cell stage at the time of the stress [4,17,19], and one of this sensitive windows is the epididymal maturation. We reported an increase in the DNA fragmentation in sperm cells that were at their transit through the epididymis (epididymal maturation) at the moment of the acute exposure to single doses of DZN [4] or MePa [17]. This unique event of spermatogenesis, chromatin condensation (when histones are replaced by protamines and disulfide bond formation occurs, which include late-spermatids and maturing spermatozoa), is at high risk of genetic damage due to the lack or reduced mechanisms of defense such as DNA-repair capacity [38] and antioxidant enzymes [20]. Similarly, Sega [19] reported that alkylation of nuclear protamines in late-spermatids and early spermatozoa eventually produce DNA breakage and compromise fertility. Thus, the DNA fragmentation observed in this study in mature spermatozoa after the exposure to different OP and their oxon metabolites may be similar to acute high occupational exposures in men during high spraying seasons, putting at risk their fertility or embryo development if workers fertilize an oocyte at the time of handling these pesticides.

Among the potential mechanisms of OP genotoxicity are the induction of oxidative stress, and several studies have addressed that oxidative damage is involved in the toxicity of pesticides, including OP, in animal studies [39], in vitro experiments [40] and in pesticide manufacturing workers [41] or pesticide sprayers [42,43]. One possible origin of oxidative stress (ROS generation) is through the CYP activation during OP metabolism, producing mainly superoxide [44]. In this regard, we previously reported that oxidative damage caused by acute exposures to MePa is involved in the DNA damage observed in epididymal mice spermatozoa [17], and Debnath and Mandal [45] reported that quinalfos exposure caused testicular damage due to LPO, along with alterations in some antioxidant enzymes and reduced levels of glutathione. Although the generation of oxidative damage by OP is a very plausible mechanism of their toxicity on sperm DNA, our results suggest that the active oxons also exert genotoxic effects probably by an oxidative mechanism. Studies are in progress to evaluate this hypothesis. Therefore, as a first approach to evaluate the mechanism of genetic damage caused by OP exposure, we evaluated the genotoxic potential of several oxon metabolites from OP with different grades of toxicity: MePA (extremely toxic) and CPF and DZN (moderately toxic).

In summary, our results strongly suggest that the oxon metabolites of OP have the ability to alter the sperm DNA integrity, even at a higher degree than their parent compounds. This effect is a plausible mechanism of OP genotoxicity in sperm cells that is worthy to be further investigated after in vivo exposures.

Conflict of interest statement

None.

Acknowledgments

Authors want to thank Víctor Rosales for his technical assistance with the flow cytometer. This study was financially supported by CONACyT-Mexico (Grant #44643) and ESA had a scholarship from this Grant.

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