Epigenetic modulation of Nrf2 and Ogg1 gene expression in testicular germ cells by methyl parathion exposure

Hernandez-Cortes D¹#, Alvarado-Cruz I¹, Solís-Heredia MJ¹, Quintanilla-Vega B¹*

¹Department of Toxicology, Cinvestav, Mexico City, Mexico, 07360.

*Corresponding Author: Betzabet Quintanilla-Vega, Department of Toxicology, Cinvestav, Ave. IPN 2508, Col Zacatenco, Mexico City, Mexico, 07360. Phone (52) +5557473310. E-mail: mquintan@cinvestav.mx

#Current affiliation address: Department of Pharmacology and Toxicology, College of Pharmacy, The University of Arizona. 1703 E. Mabel St., Tucson, Arizona, USA. 85721. E-mail: danielhc@email.arizona.edu

Running title: Epigenetic modulation as a novel mechanism of methyl parathion toxicity
**List of abbreviations**

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>AChE</td>
</tr>
<tr>
<td>Antioxidant response element</td>
<td>ARE</td>
</tr>
<tr>
<td>Base excision repair</td>
<td>BER</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>CYP</td>
</tr>
<tr>
<td>DNA methyltransferase</td>
<td>DNMT</td>
</tr>
<tr>
<td>Fetal bovine serum</td>
<td>FBS</td>
</tr>
<tr>
<td>Methyl parathion</td>
<td>Me-Pa</td>
</tr>
<tr>
<td>Nuclear factor erythroid 2-related factor 2</td>
<td>Nrf2/NRF2</td>
</tr>
<tr>
<td>Organophosphate</td>
<td>OP</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
</tr>
<tr>
<td>Room temperature</td>
<td>RT</td>
</tr>
<tr>
<td>5-hydroxymethylcytosine</td>
<td>5-hmC</td>
</tr>
<tr>
<td>5-methylcytosine</td>
<td>5-mC</td>
</tr>
<tr>
<td>5,5′-dithiobis-2-nitrobenzoic acid</td>
<td>DTNB</td>
</tr>
<tr>
<td>8-hydroxy-2′-deoxyguanosine</td>
<td>8-OHdG</td>
</tr>
<tr>
<td>8-oxo-2′-deoxyguanosine</td>
<td>8-oxo-dG</td>
</tr>
<tr>
<td>8-oxoguanine DNA glycosidase</td>
<td>Ogg1/OGG1</td>
</tr>
</tbody>
</table>
Abstract

Methyl parathion (Me-Pa) is an oxidizing organophosphate (OP) pesticide that generates reactive oxygen species (ROS) through its biotransformation. Some studies have also suggested that OP pesticides have the capacity to alkylate biomolecules, including DNA. In general, DNA methylation in gene promoters represses transcription. NRF2 is a key transcription factor that regulates the expression of antioxidant, metabolic and detoxifying genes through the antioxidant response element (ARE) situated in promoters of regulated genes. Furthermore, DNA repair genes, including 8-oxoguanine DNA glycosidase (OGG1), have been proposed as NRF2 target genes. Me-Pa exposure produces poor semen quality, genetic and oxidative damage in sperm cells, and reduced fertility. However, the Me-Pa effects on the methylation status and the expression of antioxidant (NRF2) or DNA repair (OGG1) genes in male germ cells have not been investigated. Therefore, mice were exposed to Me-Pa to evaluate the global (%5-mC) and specific methylation of Nrf2 and Ogg1 genes using pyrosequencing, gene expression, and total protein carbonylation in male germ cells. The results showed that Me-Pa significantly decreased the global DNA methylation pattern and significantly increased the methylation of two CpG sites within Ogg1 promoter and one CpG site within Nrf2 promoter. In addition, Ogg1 or Nrf2 expression did not change after Me-Pa exposure despite the oxidative damage produced. Altogether, our data suggest that Me-Pa toxicity alters Ogg1 and Nrf2 promoter methylation in male germ cells that may be modulating their gene expression.

Keywords: methyl parathion, DNA methylation, sperm cells, epigenetics, gene expression, oxidation.
Introduction

Exposure to OP pesticides is characterized by inhibition of the acetylcholinesterase (AChE) enzyme in the nervous system (Edwards and Tchounwou, 2005), but other studies have demonstrated the ability of OP pesticides to produce detrimental effects on the male reproductive system including poor semen quality, hormone imbalance, decreased fertilization rate, and impairment of embryo development (Joshi et al., 2003; Narayana et al., 2006).

Methyl parathion (Me-Pa; O,O-dimethyl O-4-p-nitrophenyl phosphorothioate) is an OP compound still utilized in many countries, even though it is classified as highly or extremely toxic by international authorities (EPA, 1986; WHO, 1993). Me-Pa has been associated with genotoxic and mutagenic effects in cells, producing chromosome aberrations and generating micronuclei (Malhi and Grover, 1987; Vijayaraghavan and Nagarajan, 1994). In particular, our group has demonstrated that Me-Pa causes genetic damage in sperm cells at different stages of spermatogenesis (Piña-Guzmán et al., 2006). Furthermore, we have previously reported that oxidative stress is the main mechanism of Me-Pa toxicity due to the generation of reactive oxygen species (ROS) during its biotransformation, which target mature and immature male germ cells (Piña-Guzmán et al., 2009). In addition, epidemiological studies have reported poor semen quality, DNA fragmentation, and sperm aneuploidies after exposure to OPs, such as Me-Pa (Padungtod et al., 1999; Recio et al., 2001; Sánchez-Peña et al., 2004).

NRF2 is a transcription factor that promotes the antioxidant response element (ARE)-mediated expression of protective and detoxifying enzymes by binding to the promoter region (Ma, 2013). Mutational assays revealed that GTGACA***GC is the ARE consensus sequence (Rushmore et al., 1991; Xie et al., 1995). Furthermore, nearby sequences and other elements influence the ARE-mediated expression and induction of detoxifying genes (Prestera et al., 1993;
Wasserman and Fahl, 1997; Xie et al., 1995). 8-oxoguanine glycosylase (OGG1) is an enzyme that belongs to the base excision repair (BER) pathway responsible for repairing oxidative modifications in nitrogenous bases damaged by ROS, including 8-oxo-deoxyguanosine (8-oxo-dG), one of the most abundant and well characterized pre-mutagenic lesions in DNA (Klunglanda and Bjellandb, 2007).

In addition to oxidative stress, OP compounds and particularly Me-Pa have shown the capacity to generate methylated adducts in guanine, adenine and cytosine in different organs, such as the testes of mice exposed to mixtures or single doses of OP compounds (Dedek et al., 1984; Segerback, 1981; Wiaderkiewicz et al., 1986), which suggests that OP pesticides have the capacity to modify the genome methylation status.

DNA methylation is an epigenetic mechanism present in the genome as 5-methylcytosine (5-mC) in critical sections close to regulatory elements in the promoter called CpG islands. In general, gene promoter hypermethylation is associated with gene silencing, while promoter hypomethylation is related to gene expression. Global genomic hypomethylation has also been associated with chromosomal instability, aging, and chronic diseases, including cancer (Millar et al., 2005). Interestingly, several studies have identified that OP compounds as well as other environmental toxicants modify the epigenetic state through DNA methylation alterations in different genes (Collotta et al., 2013).

Due to the epigenome undergoes extensive reprogramming throughout fetal development in gametogenesis and early embryo preimplantation, these stages are susceptible to environmental exposures. During gametogenesis, a genome-wide demethylation occurs in the primordial germ cells to erase previous paternal imprinting, followed by a re-methylation to
reestablish sex-specific imprinting by a meiotic cellular division (Dolinoy et al. 2007).

Regarding DNA methylation, this epigenetic mechanism plays an important role in male fertility due to its contribution to many processes during the early and late stages of spermatogenesis.

Thus, aberrant epigenetic reprogramming of male germ cells could result in alterations, ranging from poor semen quality to infertility (Dada et al., 2012). Limited information regarding the methylation capacity of OP compounds is available; specifically, alterations in methylation at global and promoter-specific levels of antioxidant and DNA repair genes, and their influence on gene expression caused by in vivo exposure to OP pesticides have not been determined. Therefore, we evaluated the effect of Me-Pa on DNA global and promoter-specific methylation and the gene expression of the DNA repair and antioxidant genes Ogg1 and Nrf2, respectively, in male germ cells from mice exposed to the pesticide.

Material and methods

Chemicals

Chemical grade (99 % purity) Me-Pa was obtained from Chem Service (West Chester, PA).

Trypsin-EDTA (0.25 %), and fetal bovine serum (FBS) was obtained from Gibco (ThermoFisher Scientific; Carlsbad, CA). Bovine serum albumin (BSA), collagenase II, hyaluronidase IS, DNase I, Nile red, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Methylated DNA Quantification Kit was acquired from Epigentek (Farmingdale, NY), and EZ-96 DNA Methylation Kit for non-methylated cytosine conversion was purchased from Zymo Research (Irvine, CA). GoTaq Hot Start Polymerase and Green Master Mix were purchased from Promega (Madison, WI). Reagents and primers for pyrosequencing were
acquired from Qiagen Inc. (Hilden, Germany). TaqMan 20X probes, Master Mix, and reagents for gene amplifications were obtained from Applied Biosystems (Foster City, CA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). RIPA and extraction buffers, halt protease and phosphatase inhibitor cocktail, and the Pierce BCA Protein Assay Kit were purchased from ThermoFisher Scientific (Carlsbad, CA). All other reagents, including those for protein blotting, were molecular grade.

Animals

Adult male ICR-CD1 mice (13-15 weeks old) were obtained from our institutional animal facilities. They were housed in filtered cages and maintained under a 12-h dark-light cycle with food and water available ad libitum. Me-Pa was dissolved in α-tocopherol-free corn oil and administered (ip) at repeated weight-adjusted doses of 6 mg/kg for 5 days, while control rodents only received the vehicle during the same period (n= 6 control and 10 treated mice). This scheme of treatment represents an occupational exposure in agricultural fields in developing countries. All animal procedures were approved by the Institutional Animal Care and Use Committee (CICUAL, for its initials in Spanish) in compliance with international guidelines for the use and care of laboratory animals.

Germ cell isolation

Mice were euthanized 24 h after the last Me-Pa administration. Testes were dissected, and the capsule was removed to obtain the seminiferous tubules that were subsequently mechanically dissected in ice-cold serum-free RPMI-1640 medium (Gibco, ThermoFisher Scientific; Carlsbad, CA). Then, seminiferous tubules were treated with a mixture of enzymes,
collagenase II (2 mg/ml), hyaluronidase IS (10 mg/ml) and DNase I (1 mg/ml) (Sigma-Aldrich, St. Louis, MO), to remove the interstitial, myoid and Leydig cells. Later, 0.25 % trypsin-EDTA was added to dissociate seminiferous tubules, releasing Sertoli and germ cells. Finally, DNase I (1 mg/ml) was added again to reduce viscosity and cell aggregation of the final suspension (Boucheron and Baxendale, 2012). After the cellular suspension was obtained (containing Sertoli and germ cells mostly), it was incubated with 0.25 µg/ml Nile red for 15 min at room temperature (RT). This compound has a high affinity for lipid droplets primarily found in the phagosomes of Sertoli cells. Then, purification of germ cells was performed using a cell sorter (MoFlo XPD High-Speed Cell Sorter; Beckman Coulter): positive Sertoli cells for Nile red were discarded and germ cells were isolated through sorting. Finally, germ cells were recovered and counted, and their characterization was indirectly performed through the absence of GATA-4, a specific marker for Sertoli cells (Riboldi et al., 2012).

**Immunocytochemistry**

Isolated germ cells were fixed with methanol for 40 min and 1 % BSA to block unspecific sites was added, and then they were incubated for 45 min at RT with 0.1 % Triton X-100 and 10 % FBS for permeabilization. Later, cells were incubated with primary anti-GATA-4 antibody (1:1200) (Rabbit monoclonal; Abcam Inc., Cambridge, MA) overnight at 4 ºC. The next day, the cells were washed and incubated with the secondary antibody (1:2000) (Goat Anti-Rabbit IgG-Alexa Fluor 488; Abcam Inc., Cambridge, MA) for 1 h at RT. Finally, the data from approximately 5,000 events were collected using an LSR Fortessa Flow Cytometer (Becton
Dickinson, Mountain View, CA). HepG2 cells were used as a positive control for the assay under the same conditions.

RNA extraction, reverse transcription and real-time RT-PCR for Ogg1 and Nrf2

Total RNA was isolated from 7x10^6 male germ cells using the TRIzol reagent. RNA quantification and purity were assessed, and integrity was determined on a 1% agarose gel. Aliquots of 30 μg of RNA were converted to cDNA using 1 μl of M-MLV reverse transcriptase following the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Real-time PCR was conducted using TaqMan probes for Ogg1 (Mm00501781_m1), Nrf2 (Mm00477784_m1), and β-actin (Mm00607939_s1). PCR reactions were performed using 0.625 μl Taq Man probe, 250 ng cDNA template, 6.25 μl TaqMan Gene Expression Master Mix, and 3.625 μl nuclease-free molecular grade water. Reactions were run in two independent experiments in triplicate. Real-time PCR was conducted using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

DNA extraction and ELISA for global DNA methylation patterns

For DNA isolation, 8x10^6 testicular germ cells were incubated with lysis buffer (0.32 M Sucrose, 5 mM MgCl_2 6H_2O, 10 mM Tris HCl, 1% Triton X-100), and then the samples were washed with suspension buffer (0.152 M NaCl, 60 mM EDTA, 34.7 mM SDS, and 0.4 M Tris-HCl) and 5 M sodium perchlorate to denature proteins. Finally, DNA was extracted with chloroform, precipitated with absolute ethanol, and washed with 70% ethanol. Once isolated, the quantity
and purity of DNA were assessed, and the integrity was determined on a 1 % agarose gel. DNA was aliquoted in nuclease-free molecular grade water at a concentration of 13.3 ng/µl for the ELISA analysis and 50 ng/µl for pyrosequencing. The relative quantification of 5-mC global content was measured using the fluorometric assay in the MethylFlash Methylated DNA Quantification Kit (Epigentek Group Inc., Farmingdale, NY) according to the manufacturer’s protocol. Aliquots of 100 ng of DNA per well were placed in microplates, and colorimetric quantification was performed at 450 nm. Two independent experiments were run and analyzed in duplicate. Positive (containing 50 % 5-mC) and negative (containing 50 % cytosine) control primers were included in the kit. A standard curve was generated by plotting different concentrations of the positive control supplied in the kit, showing a linear response in the range of 0.5 – 5.1 % 5-mC with an $R^2 = 0.9974$.

**Pyrosequencing for DNA methylation patterns of the Ogg1 and Nrf2 promoters**

DNA methylation analysis was performed using the highly quantitative bisulfite-PCR pyrosequencing assay. To select the CpG sites on Ogg1 and Nrf2 genes, an *in silico* analysis of Ogg1 and Nrf2 genes was performed. We used two web-based tools to predict NRF2-binding sites: ConSite ([http://consite.genereg.net](http://consite.genereg.net)) and Alggen ([http://wwwlsi.upc.es/~alggen](http://wwwlsi.upc.es/~alggen)). Specific criteria for regions selection were: sequences containing an ARE (NRF2-binding site) that modulates the transcription of these genes, located inside the promoter of each gene, within a CpG island, close to regions where transcriptional factors and repressors bind, and histone posttranslational modifications (H3K27Ac) have been found; avoiding polymorphisms and repetitive sequences to improve the specificity. Specifically, we chose sections right before
the transcription start site of *Nrf2* and *Ogg1* genes. According to these requirements, two short target sequences were selected on *Ogg1* containing three and five CpGs, respectively, and one target sequence was chosen on *Nrf2* containing three CpGs (Fig. 1). To determine whether these sequences were optimal for the identification of methylation using pyrosequencing, the custom PyroMark® CpG assay was used. Then, specific primers for PCR and pyrosequencing were designed using the PyroMark® Assay Design software version 2.0.1 (Qiagen, Hilden, Germany) to target multiple CpG sites for each selected sequence.

For pyrosequencing, 1 µg of genomic DNA was bisulfite-treated using the EZ DNA Methylation Gold Kit and eluted in a final volume of 40 µl according to the manufacturer’s instructions. Then, 1 µl of bisulfite-converted DNA was amplified by PCR using the GoTaq Hot Start Polymerase, and specific primers for each region were designed for bisulfite-converted DNA sequences. PCR was carried out using 15 µl of GoTaq Green Master Mix, 10 pmol forward and 10 pmol reverse primers, 50 ng of bisulfite-treated DNA, and water to reach 30 µl final volume. PCR products were purified, and CpG methylation was evaluated using 0.3 µM of sequencing primers. Pyrosequencing was performed using the PyroMark® Q24 pyrosequencing system according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Samples were run in duplicate; non-CpG cytosines present in the analyzed sequences as well as positive (containing 50 % 5-mC) and negative (containing 50 % cytosine) primers were used as pyrosequencing controls. In addition, thymines were added intentionally along the run to determine the efficiency of bisulfite conversion. The global methylation was expressed as the percent of 5-mC (%5-mC) using the PyroMark® Analysis software version 2.0.7 (Qiagen, Hilde, Germany). Samples were run in two independent experiments. Table 1
shows the target sequences and their location, the locations of PCR and biotinylated-sequencing primers, and the gene amplicon lengths.

**Protein carbonylation**

To determine protein oxidation, $10 \times 10^6$ sperm cells were isolated by flow cytometry and then lysed with RIPA buffer plus halt protease and phosphatase inhibitor cocktail previously mixed. Briefly, protein samples were quantified using the Pierce BCA Protein Assay Kit and treated for derivatization of the carbonyl groups using hydrazine. Derivatized samples were separated in a 12 % SDS-PAGE gel at 100 V for 120 min, and then proteins were transferred to a 0.45-µm nitrocellulose membrane in a semi-dry chamber for 90 min at 18 V. Later, the membrane was incubated with blocking solution overnight at 4 °C, and the next day, it was treated with the primary antibody anti-2,4-dinitrophenylhydrazone (DNPHz) (1:5000) for 2 h. The membrane was rinsed with TBS buffer and then incubated with the secondary antibody goat anti-rabbit IgG H&L (1:8000) for 2 h. Finally, the membrane was washed again and developed with the ECL Prime Western Blotting Detection Kit (GE Healthcare Life Sciences, Pittsburgh, PA) following the manufacturer’s instructions.

**Erythrocyte acetyl cholinesterase (AChE) activity**

Peripheral blood samples were obtained by laceration of the tail artery 24 h after the last Me-Pa administration and collected in heparinized vials. AChE activity was determined by the hydrolysis of acetylthiocholine iodide following Ellman’s method (Ellman et al., 1961) using
thiocoline as a substrate, quinidine sulfate as a plasma cholinesterase inhibitor, and 5,5'-
dithiobis-2-nitrobenzoic acid (DTNB). Samples were detected at 412 nm each minute for 6 min
at RT. The AChE activity was expressed as µmol thiocholine min⁻¹ ml⁻¹.

Statistical analyses

The data from the control and exposed groups were compared by the two-sided Wilcoxon rank
sum test using STATA/IC software version 15.0 for Mac (StataCorp, College Station, TX).
Statistical significance was assigned at a p value ≤ 0.05.

Results

General toxicity

The common neurological signs associated with OP poisoning were observed in mice during
the administration of Me-Pa (6 mg/kg/day/5 days), and the animals recovered after 3 h of the
daily exposure. In addition, Me-Pa exposure was evaluated by the AChE activity 24 h after the
last administration, which was significantly inhibited to 66 % of its activity (p < 0.01), with values
of 1.204 ± 0.090 µmol thiocholine min⁻¹ ml⁻¹ in exposed mice compared to 1.816 ± 0.047 µmol
thiocholine min⁻¹ ml⁻¹ in the control group.

Germ cell isolation and immunocytochemistry
The extraction of male germ cells from mice was performed by cell sorting via flow cytometry using the fluorochrome Nile red as a tracker. The cell population was first distributed by size (FSC-Height) and granularity (SSC-Height) to discard cellular debris, selecting the R1 region (Fig. 2A). Then, cells within this region were redistributed by size (FSC-Height) and fluorescence intensity (FL2-Log-Height) to isolate germ cells through sorting of the R2 region (Fig. 2B). Purity of isolated germ cells was analyzed by the presence of Sertoli cells using the GATA-4 marker. The immunodetection of Sertoli cells was assessed before (2.355 ± 0.240 fluorescence units) and after (0.020 ± 0.012 fluorescence units) sorting, obtaining a final purity of 99% germ cells. HepG2 cells were used as a positive control for the immunodetection of GATA-4 (Fig. 2C).

mRNA expression levels of Ogg1 and Nrf2 in germ cells

The mRNA levels of the Nrf2 and Ogg1 genes were evaluated to determine whether Me-Pa treatment was capable of changing their expression. No difference (p > 0.05) in Ogg1 gene expression between the control (1.543 ± 0.076) and treated (1.551 ± 0.175) groups was found (Fig. 3A), suggesting a lack of induction. Likewise, although reaching borderline significance, a non-significant difference (p = 0.0509) in Nrf2 gene expression between the control (1.001 ± 0.104) and treated (1.289 ± 0.092) groups was observed (Fig. 3B).

Global and specific methylation patterns in germ cells
An ELISA kit containing the primary antibody against 5-mC was used to determine the global methylation status of the genome. Levels of 5-mC were significantly decreased ($p < 0.05$) in mice exposed to the pesticide ($1.411 \pm 0.132$) compared to control mice ($2.059 \pm 0.242$), and an approximately 32% reduction in global DNA methylation was observed (Fig. 4).

In addition to global DNA methylation, the specific methylation within the promoters of $Ogg1$ and $Nrf2$ after Me-Pa exposure was determined. Sequenced sections were selected for convenience (see Methods Section) assuming that these regions are critical sites that regulate the expression of the two evaluated genes. For the $Ogg1$ promoter, two regions were selected, while only one region of the $Nrf2$ promoter was chosen. From the three CpG sites that were located in the first region of $Ogg1$, a significant difference in the first ($p < 0.01$) and second ($p < 0.05$) CpG sites was observed in Me-Pa-treated animals compared to control mice (Figs. 5A-B). However, no difference ($p > 0.05$) was observed in the third CpG site between groups (Fig. 5C).

In addition to the comparison between individual CpG sites, the mean value of %5-mC of all CpG sites per target region of each gene was also compared between groups. A significant difference ($p < 0.01$) in the mean value of the three CpG sites of the first region of $Ogg1$ between control and exposed mice was observed (Fig. 5D). Regarding the second region sequenced in the $Ogg1$ promoter, five CpG sites were evaluated. However, none of these sites was significantly different ($p > 0.05$) between the treated and control groups (Figs. 6A-E). Similarly, there was no difference ($p > 0.05$) in the mean values of all five CpG sites of the second region of $Ogg1$ between the treated and control animals (Fig. 6F).

The methylation pattern of the $Nrf2$ promoter was determined in one region containing three CpG sites. No differences ($p > 0.05$) in the first CpG site (Fig. 7A) or in the second CpG site (Fig. 7B) between the exposed and control groups were observed. However, we observed a
significant difference ($p<0.05$) in the third CpG site (Fig. 7C) between the treated and control mice. Finally, there was no difference ($p>0.05$) in the mean values of the three CpG sites between the treated and control groups (Fig. 7D).

Protein carbonylation

We evaluated the carbonylation of total protein of male germ cells by immunodetection as a broad indicator of protein oxidation in our model. Mice exposed to Me-Pa showed a significant increase of 44% in protein oxidation compared to control mice ($p<0.05$) (Fig. 8).

Discussion

We identified for the first time alterations in the epigenetic status of male germ cells at both the global and promoter-specific levels caused by in vivo exposure to an OP pesticide as a novel mechanism of toxicity that might modulate Ogg1 and Nrf2 gene expression. OPs are highly toxic pesticides of restricted use or banned by the European Union (Pesticide Action Network, 2008). In the United States, OP pesticides accounted for 33% (20 millions of pounds) of all insecticide products used nationwide in 2012 (EPA, 2012), and their use in developing countries, such as Mexico, is still a health public concern (Secretaría de Salud, 2017).

In this study, repeated doses of Me-Pa decreased the global DNA methylation profile of testicular germ cells. Tunc and Tremellen (2009) reported a negative correlation between DNA methylation and DNA fragmentation as well as ROS production in sperm from infertile men, suggesting that oxidative damage to sperm DNA impairs the methylation of cells. We did not
measure DNA damage or oxidative stress generation after Me-Pa exposure in this study. However, our group has reported that the oxidative capacity of Me-Pa is involved in sperm DNA damage, compromising the fertilization rate of germ cells (Piña-Guzmán et al., 2006; 2009; Salazar-Arredondo et al., 2008). Therefore, the significant decrease in global DNA methylation observed in this study could be related to the oxidative stress generated by Me-Pa exposure; nevertheless, more studies are needed to determine whether the decrease in the fertilization capacity is a consequence of the global hypomethylation promoted by Me-Pa oxidative stress in male germ cells. Biotransformation of OP pesticides through cytochrome P450 (CYP) enzymes has been reported in the testis (Moustafa et al., 2008), and CYP-mediated reactions generate ROS through redox-cycling activity (Bondy and Naderi, 1994), supporting that OP-induced male reproductive toxicity arises from in situ metabolism in the testis.

Two central cellular defense mechanisms against oxidative damage are the antioxidant systems and the DNA repair enzymes. In this study, a slight but non-significant increase in the expression of Nrf2 and no change in Ogg1 expression were observed. Apparently, Me-Pa exposure was not sufficient to induce either Ogg1 or Nrf2 despite the presence of oxidative damage detected as protein carbonylation in male germ cells from mice. Literature regarding the effect of OP pesticides on the expression of genes involved in antioxidant or DNA repair mechanisms is scarce; nonetheless, a reduction in the expression of genes and proteins related to DNA repair, antioxidant response, and cellular metabolism after in vitro (Mankame et al., 2006) or in vivo (Chen et al., 2015) exposure to OP pesticides has been reported. In addition, even though OGG1 has not been reported as a downstream gene of NRF2, silencing of NRF2 downregulates OGG1 protein levels and reduces OGG1 gene
activity because an ARE is located within the \textit{OGG1} promoter (Habib et al., 2016; Singh et al., 2013), which could explain the lack of \textit{OGG1} gene induction.

We evaluated gene-specific DNA methylation because there is vast literature suggesting that environmental exposures can produce changes on different epigenetic mechanisms, altering gene expression as consequence. In addition, CpG islands have been associated to housekeeping gene promoters and this characteristic is shared in both species; mouse and humans (Antequera & Bird, 1993). An autonomous CpG site methylation level has been proposed (Pfeifer et al., 1990), but the specific mechanisms that regulate this process are not well understood.

Regarding specific methylation, we found that repeated doses of Me-Pa increased the methylation of specific CpG sites within the \textit{Nrf2} and \textit{Ogg1} promoters. Even though germ cells from all stages of spermatogenesis are found in the seminiferous tubules, we most likely collected a homogeneous population of postmeiotic germ cells. However, since methylation changes occur less in these cells, particularly during spermiogenesis (Oakes et al., 2012), we suggest that the hypermethylation observed by repeated exposure to the pesticide (control vs treated) may be due the ability of Me-Pa to methylate \textit{per se} the DNA (Wiaderkiewicz et al., 1986).

Information regarding promoter-specific methylation changes promoted by exposure to OP pesticides is very limited. However, some OPs, including fonofos, parathion, and terbufos, induced methylation in the promoters of genes related to DNA damage and repair, among others, in human hematopoietic K562 cells (Zhang et al., 2012a). Furthermore, the OP pesticide diazinon was capable to methylate promoters of tumor suppressors and other related
genes related to the hallmarks of cancer due to its alkylating ability in K562 cells, suggesting the potential role of OP pesticides in cancer development via epigenetic modifications (Zhang et al., 2012b).

We observed a global hypomethylation and promoter-specific hypermethylation in male germ cells. It is not surprising to observe global DNA hypomethylation and hypermethylation in specific genes, this is a phenomenon commonly described in cancer development (Fotouhi et al., 2014), including prostate cancer (Cho et al., 2006), and it was recently reported in pesticide applicators exposed to a mixture of pesticides (Rusiecki et al., 2017). Currently, there is no clear mechanism that could explain the occurrence of both hypomethylation and hypermethylation events at the same time, but these phenomena could be caused by independent mechanisms (Roman-Gomez et al., 2006) that require being further investigated.

We observed that some CpG sites within the Ogg1 and Nrf2 promoters were significantly methylated, while the expression levels of these genes were not significantly increased as expected due to the presence of oxidative damage after Me-Pa treatment. In other words, since we have previously shown the oxidative capacity of Me-Pa (Piña-Guzmán et al., 2006), we hypothesized that Nrf2 and Ogg1 should have expressed to repair this damage. By not detecting increases in their mRNA levels we suggest that the pesticide altered Nrf2 and Ogg1 expression. The relationship between promoter DNA methylation and downregulation of Nrf2 (Huang et al., 2012) and OGG1 (Singh et al., 2012) gene expression has been reported in mice and human MCF-7 cells, respectively.

Likewise, this correlation has also been described in epidemiological studies performed in breast cancer patients for OGG1 (Fleischer et al., 2014) and in prostate cancer patients for
Interestingly, despite the differences in length and number of CpG sites between the human and mouse NRF2 gene promoters, these authors studied the methylation of a region in NRF2 with the same characteristics as those selected in our study: a section located within the promoter and right before the transcription start site and the first exon of the gene. In human testis, NRF2 protects the disruption of spermatogenesis caused by oxidative stress, low Nrf2 expression causes high ROS levels and defective spermatogenesis that predispose individuals to infertility (Nakamura et al., 2010; Chen et al., 2012). Hence, we suggest that this region within the Nrf2 gene could be an important target site for epigenetic regulation. However, further in vitro studies regarding regulation of Nrf2 gene expression are needed to evaluate the effect of methylation in the CpG sites evaluated in our study.

As a single phenomenon, only a few studies have simultaneously evaluated DNA methylation and oxidation. However, they have suggested that the oxidation of either a single guanine into 8-oxoguanine (8-oxo-G) or 8-oxo-dG or 5-mC into 5-hydroxymethylcytosine (5-hmC), both adjacent to CpG sites, is crucial for the binding of proteins involved in the condensation and inactivation of chromatin (Valinluck et al., 2004) and for the binding of transcription factors to DNA (Zawia et al., 2009). Moreover, the last group and Kasymov et al. (2013) reported that the methylation of a cytosine adjacent to an oxidized guanine (8-hydroxy-2′-deoxyguanosine, 8-OHdG) in CpG sites not only synergistically decreases Ogg1 gene expression but also impairs the ability of OGG1 protein to repair the oxidative damage. Finally, ROS might induce site-specific hypermethylation via the upregulation of DNMT expression or by increasing the formation of DNA methyltransferase (DNMT) complexes (Wu and Ni, 2015). As an oxidative compound, Me-Pa may increase the methylation of Ogg1 and Nrf2 promoters by these mechanisms, which needs further investigation.
mechanism involved in the interaction between DNA methylation and oxidative stress is not fully understood and needs to be elucidated.

In our study, we did not measure DNA oxidation caused by Me-Pa exposure. However, evidence from our group has shown the oxidative capacity of repeated doses of Me-Pa in mice through the presence of 8-OHdG in DNA from spermatozoa (Monroy-Pérez et al., 2012) and carbonylation of total protein in testis homogenate (Tello-Mora, In preparation).

In summary, our study demonstrates that Me-Pa is capable of inducing methylation changes in gene promoters of DNA repair and antioxidant pathways, which resulted in the absence of gene induction. We suggest that gene silencing of Ogg1 and Nrf2 impaired the antioxidant capacity as well as the DNA repair machinery in germ cells, which could contribute to the toxicity of the pesticide. Further studies are needed to elucidate the mechanism by which Me-Pa and potentially other OP pesticides change the epigenetic landscape of male germ cells, whether OP compounds directly alkylate the DNA bases or disturb the methylation machinery by increasing ROS levels. Furthermore, the role that methylation changes might play in the development of detrimental effects on semen quality and fertility, as well as the biological consequences of aberrant germ cell epigenetic marks after OP pesticide exposure, also require additional investigation.

**Acknowledgments.** The authors want to thank Víctor Rosales-García for his assistance with cell sorting, Gerardo Martínez-Aguilar for technical assistance, and Dr. Juliana Navarro-Yepes for her valuable help with the protein carbonylation assay. DHC was a recipient of a scholarship from CONACYT (National Council of Science and Technology)-Mexico.

**Funding sources**
The researchers did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Conflict of interest**

The authors declare no conflicts of interest.

**References**


PAN. Pesticide Action Network, 2008. Which Pesticides are Banned in Europe? Available at: http://www.pan-europe.info/old/Resources/Links/Banned_in_the_EU.pdf


hypermethylation and global hypomethylation are independent epigenetic events in lymphoid leukemogenesis with opposing effects on clinical outcome. Leukemia, 20(8), 1445–1447.


Table 1. Selected regions of the \textit{Nrf2} and \textit{Ogg1} gene promoters for pyrosequencing.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>Chromosome location</th>
<th>PCR and sequencing primer</th>
<th>Target sequence</th>
<th>Number of CpGs</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>chr6: 113,326,873 – 113,326,908</td>
<td>F:GGTGGGAGTA AATTGGG R:ACCACCAAAC CCCTAAAC</td>
<td>CCCGAAGAAC CATACTGGCGC GCCTTTCAAAC</td>
<td>3</td>
<td>176</td>
</tr>
<tr>
<td>\textit{Ogg1}</td>
<td>Second</td>
<td>chr6: 113,326,956 – 113,326,993</td>
<td>F:GGTGGGAGTA AATTGGGATT R:CACACCACCA AACCCCTAA</td>
<td>CCGGCCACGT CCGAGGAAGA GGGGGGGCGT GCTGCA</td>
<td>5</td>
<td>79</td>
</tr>
<tr>
<td>\textit{Nrf2}</td>
<td>First</td>
<td>chr2: 75,704,799 – 75,704,832</td>
<td>F:TAAGGGGAG GGGGGGATA R:ACTTCAACCC ACCTAACT</td>
<td>CCGAGGGCGG GCCATGGACCT GAGTGGTCGG GC</td>
<td>3</td>
<td>165</td>
</tr>
</tbody>
</table>

Genomic locations of the evaluated regions according to UCSC genome browser version GRCm38/mm10, Dec. 2011. The CpG sites in the target sequences are bold. Target sequences are given before bisulfite conversion.
Figure legends

Figure 1. Regions within the Ogg1 and Nrf2 promoters selected for sequencing. Location of the regions sequenced in the Ogg1 (A) and Nrf2 (B) genes according to their respective transcription start site (TSS) in the promoter. The CpG sites in the target sequences are bold.

Figure 2. Isolation of male germ cells from mice. (A) Flow cytometry of the cellular suspension from testis distributed by size and granularity. (B) Sorting of the germ cells taken from the cellular suspension and distributed by size and fluorescence. (C) Immunocytochemistry of the Sertoli cell marker GATA-4 in germ cells before and after flow cytometry. Bars represent the mean ± SEM of 2 independent experiments. Significant difference (*p < 0.05) between groups according to the Wilcoxon rank-sum test. HepG2 cells were used as a control for GATA-4 expression.

Figure 3. mRNA expression levels of Ogg1 (A) and Nrf2 (B) in male germ cells after Me-Pa exposure. Bars represent the mean ± SEM of 2 independent experiments performed in triplicate. The data were normalized to actin. There was no significant difference (p > 0.05) between groups according to the Wilcoxon rank-sum test, n = 6 controls and 10 treated.

Figure 4. Global DNA methylation levels in male germ cells after Me-Pa exposure. Bars represent the mean ± SEM of 2 independent experiments. Significant difference (**p < 0.01,
*p < 0.05) between groups according to the Wilcoxon rank-sum test, n = 6 controls and 10 treated.

**Figure 5.** DNA methylation levels of CpG sites within the first Ogg1 target region in male germ cells after Me-Pa exposure. Methylation of the first (A), second (B), and third (C) CpG sites and the mean value across the 3 CpG sites (D). Bars represent the mean ± SEM of 2 independent experiments performed in duplicate. Significant difference (**p < 0.01, *p < 0.05) between groups according to the Wilcoxon rank-sum test, n = 6 controls and 10 treated.

**Figure 6.** DNA methylation levels of CpG sites within the second Ogg1 target region in male germ cells after Me-Pa exposure. Methylation of the first (A), second (B), third (C), fourth (D), and fifth (E) CpG sites and the mean value across the 5 CpG sites (F). Bars represent the mean ± SEM of 2 independent experiments performed in duplicate. There was no significant difference (p > 0.05) between groups according to the Wilcoxon rank-sum test, n = 6 controls and 10 treated.

**Figure 7.** DNA methylation levels of CpG sites within the Nrf2 target region in male germ cells after Me-Pa exposure. Methylation of the first (A), second (B), and third (C) CpG sites and the mean value across the 3 CpG sites (D). Bars represent the mean ± SEM from 2 independent experiments performed in duplicate. Significant difference (*p < 0.05) between groups according to Wilcoxon rank-sum test, n = 6 controls and 10 treated.
Figure 8. Densitometry analysis of total protein carbonylation in male germ cells of mice exposed to Me-Pa. Bars represent the mean ± SEM of one experiment. Significant difference (*p < 0.05) between groups according to the Wilcoxon rank-sum test, n = 4 controls and 3 treated.