Toxicology and Applied Pharmacology 230 (2008) 261–268

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

PON1Q192R genetic polymorphism modifies organophosphorous pesticide effects on semen quality and DNA integrity in agricultural workers from southern Mexico

N. Pérez-Herrera [a], H. Polanco-Minaya [a,b], E. Salazar-Arredondo [a,c], M.J. Solís-Heredia [a], I. Hernández-Ochoa [a,1], E. Rojas-García [d], J. Alvarado-Mejía [e], V.H. Borja-Aburto [f], B. Quintanilla-Vega [a,*]

[a] Sección Externa de Toxicología, CINVESTAV-IPN, PO Box 14-760, Mexico City, 07360, Mexico
[b] Facultad de Química, Universidad Autónoma de Yucatán, Mérida, Yucatán, 97150, Mexico
[c] Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Mexico City, 97150, Mexico
[d] Dirección de Fortalecimiento de la Investigación, Universidad Autónoma de Nayarit, Tepic, Nayarit, 63190, Mexico
[e] Facultad de Medicina, Universidad Autónoma de Yucatán, Mérida, Yucatán, 97000, Mexico
[f] Coordinación Nacional de Salud en el Trabajo, IMSS, Mexico City, 06725, Mexico

ARTICLE INFO

Article history:
Received 17 December 2007
Accepted 22 February 2008
Available online 4 March 2008

Keywords:
PON1 polymorphisms
Organophosphorous pesticides
Sperm DNA damage
Semen quality

ABSTRACT

Pesticide exposure, including organophosphorous (OP) insecticides, has been associated with poor semen quality, and paraoxonase (PON1), an enzyme involved in OP deactivation, may have a role on their susceptibility, due to PON1 polymorphisms. Our objective was to evaluate the role of PON1Q192R polymorphism on the susceptibility to OP toxicity on semen quality and DNA integrity in agricultural workers. A cross-sectional study was conducted in farmers with Mayan ascendancy from southeastern Mexico chronically exposed to pesticides; mostly OP. Fifty four agricultural workers (18–55 years old) were included, who provided semen and blood samples. Semen quality was evaluated according to WHO, sperm DNA damage by *in situ*-nick translation (NT-positive cells), PON1Q192R polymorphism by real-time PCR and serum PON1 activity by using phenylacetate and paraoxon. Two OP exposure indexes were created: at the month of sampling and during 3 months before sampling, representing the exposure to spermatids–spermatozoa and to cells at one spermatogenic cycle, respectively. PON1 192R and 192Q allele frequencies were 0.54 and 0.46, respectively. Significant associations were found between OP exposure at the month of sampling and NT-positive cells and sperm viability in homozygote 192RR subjects, and dose–effect relationships were observed between OP exposure during 3 months before sampling and sperm quality parameters and NT-positive cells in homozygote 192RR farmers. This suggests that cells at all stages of spermatogenesis are target of OP, and that there exists an interaction between OP exposure and PON1Q192R polymorphism on these effects; farmers featuring the 192RR genotype were more susceptible to develop reproductive toxic effects by OP exposure.

Introduction

Genetic polymorphisms as modifiers of human health diseases have received attention in the last decade, and there is an increased interest in conducting studies to explore the gene–environment interactions to detect susceptible populations prone to develop health problems by chemical exposures (Kelada et al., 2003). Recent advances in molecular epidemiology have suggested that polymorphisms on enzymes involved in the metabolism of environmental chemicals would render individual susceptibility to their toxicity. Pesticides are an important class of these environmental chemicals, and included among them are the organophosphorous (OP) insecticides that are used worldwide (IARC, 1991).

OP compounds are among the most widely used pesticides, mainly as insecticides in agriculture, health campaigns and urban pest control. In the last decade, adverse effects of OP exposure on the male reproductive system, along with their mutagenic and carcinogenic activities have attracted attention (IARC, 1991). Workers from a manufacturing plant of OP showed poor semen quality (low concentration and motility) (Padungtod et al., 2000), similar to men environmentally exposed to chlorpyrifos (an OP compound) and carbaryl (Meeker et al., 2004a). In animal models, our previous studies have shown that single doses of methyl parathion and diazinon administered to mice alter sperm quality and DNA integrity, evaluated as the percentage of cells with endogenous nicks (nick translation assay) (Piña-Guzmán et al., 2005, 2006), while Burruel et al. (2000) reported that methamidophos caused a dose–response increase in abnormal sperm morphology, as well as a decreased fertility rate and embryo degeneration in mice.

Abbreviations: PON1, human paraoxonase 1; OP, organophosphorous pesticides; 8-OHdG, 8-hydroxydeoxyguanosine; NT, nick translation; BMI, body mass index.

* Corresponding author. Sección Externa de Toxicología, CINVESTAV-IPN, Av. IPN #2508, Col. Zacatenco, Mexico City, 07360, Mexico. Fax: +52 55 5061 3395.
E-mail address: mquintan@cinvestav.mx (B. Quintanilla-Vega).
[1] Present address: Department of Veterinary Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL, 61802, USA.

0041-008X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2008.02.021

These data suggest that paternal exposure to OP has the potential to produce transmissible adverse effects to the embryo.

Most OP are organothiophosphates that require metabolic activation through oxidative desulfuration to form the corresponding oxons; this bioactivation is mediated by several cytochrome P450 (CYP) enzymes (Jokanovick, 2001). On the other hand, OP deactivation is mediated mainly by serum paraoxonase (PON1) [E.C.3.1.8.1]. Human *PON1* is a member of a multigene family (*PON1*, *PON2* and *PON3* genes) encoded by a single gene on chromosome 7q21–22 (Primo-Parmo et al., 1996). PON1 is synthesized in the liver and secreted into the blood, where it is associated with high density lipoproteins (HDL). PON1 activity in serum shows a wide variation among individuals (La Du et al., 1986), and this variability is attributed to the presence of polymorphisms in *PON1* gene; thus, subjects have been classified into homozygous for the low activity allele, and heterozygous, or homozygous for the high activity allele (Eckerson et al., 1983). At least five polymorphisms in the *PON1* promoter region have been described, in which −108 C/T contributes to about 23% of the activity (Brophy et al., 2001). Meanwhile, PON1 coding region shows two polymorphic sites at positions 55 and 192; the latter has two isoforms (Q and R) and is described as substrate-dependent, thus the 192R alloenzyme hydrolyzes paraoxon (the parathion oxon metabolite) faster than the 192Q isoform, while diazoxon (the oxon of diazinon) is hydrolyzed faster by the 192Q isoform (Davies et al., 1996).

In addition to the PON1 capacity to hydrolyze and hence inactivate various OP (Geldmacher-von Mallinckrodt and Diepgen, 1988), PON1 can prevent oxidation of LDL (low density lipoproteins) and HDL by its anti-oxidant activity (La Du, 1996). Therefore, it has been suggested that PON1 may have important roles in the development of diseases such as cardiovascular heart disease, atherosclerosis, diabetes, Alzheimer's dementia and Parkinson, and in OP toxicity (review in Li et al., 2003). In this regard, Cherry et al. (2002) showed that English farmers with ill health were more likely to have at least one 192R allele and a lower ability for the hydrolysis of diazoxon than controls; Lee et al. (2003) found a higher prevalence of chronic toxicity among pesticide applicators from South Africa featuring the 192QQ or 192QR genotypes. In addition, Haley et al. (1999) observed that Gulf War Veterans with symptoms of neurological damage were more likely to have the 192R allele (192RR or 192QR genotypes) than homozygote subjects for the 192Q allele. Similarly, Hotopf et al. (2003) observed lower PON1 activity in Gulf War Veterans and suggested that they were exposed to hazards that led to a long-term decrease in PON1 activity, among the candidates are the OP neurotoxic nerve gases. Finally, Mackness et al. (2000) suggested that the decreased capacity to detoxify OP resulting from low serum PON1 activity may have contributed to the development of the Gulf War Syndrome. On the other hand, some studies have failed to find an association between PON1 polymorphisms and OP toxicity. Padungtod et al. (1999) explored the relationship between PON1Q192R polymorphism and the effects on semen quality in Chinese pesticide factory workers exposed to ethyl and methyl parathion and methamidophos, but did not observe a gene-dose effect of PON1 genotype respect to semen quality. Meanwhile Liu et al. (2006) concluded that PON1Q192R polymorphism did not influence the leukocyte genetic damage (DNA tail moment by Comet assay) in pesticide-exposed fruit workers, which is in agreement with Lee et al. (2007) who reported that PON1 polymorphism at position 192 did not modulate DNA damage (evaluated by 8-hydroxydeoxyguanosine (8-OHdG) levels) in leukocytes and urine specimens in indoor sprayers.

Although it is known that working in agriculture increases the risk of abnormal semen quality, and that the presence of PON1 polymorphisms that confer different hydrolyzing abilities toward OP, has inspired the hypothesis that certain individuals may be more sensitive to OP toxicity, the role of PON1 polymorphisms on male reproductive toxicity caused by OP exposure is poorly studied. Considering that OP poisoning represents an important public health problem in Mexico, the aim of this study was to evaluate the role of PON1Q192R genetic polymorphism on the effects on semen quality and DNA integrity by OP exposure in Mexican agricultural workers.

Methods

Study population. A cross-sectional study was conducted in a cohort of traditional agricultural workers from southeastern Mexico, in a community from Yucatán State, during 2005. Inhabitants from this rural region have a strong Mayan ascendancy (44% speak the native Mayan language) (INEGI, 2005), with a low socioeconomic status and agriculture as lifestyle. Previous surveys conducted in this community by local physicians had shown reproductive health outcomes in agricultural farmers' wives, such as abortions, miscarriages and premature deliveries (unpublished undergraduate thesis); these antecedents raised our interest in conducting this study. Public invitations to inhabitants were done to recruit participants with the assistance of local health authorities. Eligible subjects were healthy men employed in agriculture, from 18–55 years old that were born in Yucatán State. Men with vasectomy were not included and one subject was excluded because of azoospermia. All participants signed an informed consent form. A total of 54 agricultural workers accepted to participate in the survey. The participation rate (26%) obtained in this study, the first one conducted in this rural population having a strong Mayan ascendancy, was comparable to that obtained in other epidemiological studies that have evaluated semen quality in traditional farmers or urban inhabitants from Europe and North America (Jørgensen et al., 2001; Swan et al., 2003). Sociodemographic characteristics, lifestyle habits (diet, cigarette smoking and alcohol drinking), health status, and pesticide use history were directly acquired from a structured questionnaire applied by trained personnel. This study was approved by the Ethics Committee from CINVESTAV-IPN (IRB000478-CINVESTAV IRB#-COBISH) in accordance to the Helsinki Declaration.

Sampling. Blood samples were obtained by venipuncture and collected in EDTA and heparin coated tubes to analyze PON1Q192R polymorphism and PON1 activities, respectively. Aliquots of whole blood and plasma were stored at −70 °C until analysis. Semen samples were collected by masturbation after at least 3 days of sexual abstinence into clean plastic containers provided for this purpose. Participants were instructed on how to collect the samples (washing their hands before collection and within 1 h before arrival to our provisional laboratory installed in the community). Information on date, time and number of days since last ejaculation was recorded.

Semen analysis. Semen examination was carried within 1 h of collection according to World Health Organization guidelines (WHO, 2002). After liquefaction, volume was recorded and sperm concentration and motility were evaluated by a semen analyzer (SQA, Model II-C P, Medical Electronic Systems Ltd.). Sperm viability was evaluated by eosin–nigrosin staining. Slides for morphology scoring were air-dried and stained using a modification of Papanicolaou's stain. Samples were analyzed by two trained technicians.

Nick translation assay. The nick translation method was performed according to Summer et al. (1990). The slides with sperm spread were washed in phosphate buffer solution (PBS), fixed in methanol/acetic acid (3:1) and air-dried. *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 (without DNA polymerase I) and a positive control (incubation with DNase I before addition of polymerase mixture) were included. Streptavidin (SAHR)–fluorescein isothiocyanate (FITC) was used to evidence the incorporation of biotinylated dUTP (NT-positive cells). After staining, 200 randomly selected cells were analyzed with an Olympus BX40 fluorescence microscope using 485 and 520 nm excitation and barrier filters, respectively.

PON1 genotype. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. All PCR procedures were performed under an ultraviolet-irradiated hood. PON1 genotypes were determinate by real-time PCR ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems) according to Rojas-García et al. (2005). Briefly, the primers used were CTGAGCACTTTTATGGCACAAATGA/ACCACGC-TAAACCCAAATACATCTC and the VIC/FAM probes were CCTACTTACAATCCTG/CCCTACT-TACGATCCTG. Reactions were performed with 5 μl of TaqMan Universal PCR Master Mix (containing AmpliTaq Gold DNA Polymerase, Amperase UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components), 0.25 μl of 40× primer–probe mix (containing primers at 36 μM and dye-labeled probes at 8 μM), and approximately 20 ng of genomic DNA; finally, the reaction mixture was completed to 10 μl with water. Thermal cycling conditions were as follow: a step of 50 °C for 2 min, a hot-start at 95 °C for 10 min, and a two step protocol was followed by 40 cycles: 95 °C for 15 s and 60 °C for 1 min. Allele discrimination was detected by two differential TaqMan probes labeled with the reporter dye 6-carboxyfluorescein (6-FAM) and VIC at the 5′end.

PON1 activity. PON1 enzymatic activities were assayed using paraoxon (paraoxonase activity) or phenylacetate (arylesterase activity) as described byEckerson et al. (1983) and Furlong et al. (1988). Arylesterase activity was determined by phenol production from the hydrolysis of phenylacetate (Sigma-Aldrich) using 10 mM Tris–HCl. pH 8.0, 1 mM $CaCl_2$ and 5 μM EDTA. Eserine sulfate (Sigma-Aldrich) was used to inhibit the unspecific hydrolysis due to serum albumin and serum cholinesterase. The increase

in A_{270} was followed for 5 min and the molar extinction coefficient of phenol is 1.31×10^3; arylesterase activity was expressed as U/ml. Paraoxonase activity (hydrolysis of paraoxon; ChemService) was performed in the presence of 1 M NaCl (salt-stimulated) to get better identification of PON1 phenotype (Eckerson et al., 1983). The paraoxonase activity was assayed by monitoring the *p*-nitrophenol formation. The increase in A_{405} was followed for 5 min and the molar extinction coefficient of *p*-nitrophenol is 18.05×10^3; paraoxonase activity was expressed as U/l.

Exposure assessment. Information collected from the questionnaires was designed to identify the exposure history from each worker. Two exposure indexes were constructed for all pesticides and for the group of OP: at the month of sampling (as a reflection of a recent exposure, representing the exposure to spermatids and mature spermatozoa), and during the preceding 3 months before sampling (as a refection of the exposure to cells during a complete spermatogenic cycle). The main determinants to estimate the exposure indexes were: a) exposure frequency to all pesticides, b) exposure frequency to OP, c) use of personal protection, and d) habits that may modify the exposure. The frequency of use to all pesticides or to OP (days/month) at the month of sampling or during the preceding 3 months before collecting the samples (the sum of the exposure from each month) was taken from the questionnaires. Then, a protection factor was created considering personal protection used during handling pesticides according to the absorption rates of potential exposures as follow: wearing waterproof gloves represented a 15% of protection, pants 3.5%, overalls 7%, water boots 3.5%, hat or cap 1%, mask 60%, mouth cover 10% and security lens 1%. If used, these protection percentages were subtracted from the unit, and if farmers did not use personal protection, the factor was kept as the unit. The habits (frequency) that may modify the exposure were considered as follows: washing the spraying equipment increased the exposure by 30%, storing pesticides in their homes by 60%, eating during the journal day by 30%, and eating while preparing the pesticide mixture by 30%. The only habit that decreased the exposure (30%) was taking a bath after the journal day. If farmers had any of these habits, the percentage of increment or decrement of exposure was subtracted or added to the unit. Finally, we calculated the exposure index in two steps: first, we multiplied the frequency of OP or all pesticides use by the protection factor and second, the frequency of OP or all pesticides use was multiplied by the habits factor, and finally, these two values were added to give the final exposure indexes from each worker.

Statistical analysis. Variable description and normal distribution were explored. Elevation to square and cubic root transformations of sperm morphology and viability, respectively, and logarithmic transformation of NT-positive cells, ejaculate volume and paraoxonase activity were performed to improve normality. Semen quality parameters and NT-positive cells were considered as dependent variables and PON1Q192R genotype and activities and OP exposure expressed as exposure indexes (at the month of semen collection and during the preceding 3 months before sampling) were considered as independent variables. Age, body mass index (BMI), cigarette smoking, alcohol drinking, caffeine intake, sexual abstinence days, time working in agriculture and exposure to all pesticides were considered as covariates. The chi square test was used to estimate the concordance of PON1Q192R genotype frequencies with the Hardy–Weinberg's equilibrium and to compare PON1Q192R genotype and allele frequencies with other populations. Pearson analysis was done to explore the correlation between sperm DNA integrity and semen quality parameters. Differences among PON1Q192R genotypes and paraoxonase and arylesterase activities were estimated by ANOVA test. Simple and multiple regression analysis were done to asses the association between semen quality parameters and OP exposure indexes, considering the month of semen collection and 3 months before sampling. Then, workers were separated by their genotype to explore the role of PON1Q192R genetic polymorphism as a modifier of OP effects on semen quality and DNA integrity. All statistical analysis were conducted by using the STATA Program version 8.1 (STATA, Corp.) and the significance was set as $p<0.05$.

Table 1
Demographic characteristics of agricultural workers

Characteristic	Prevalence
Age (years), mean±SD	39.2±10.2
Body mass index (kg/m2), median (range)	27.3 (21.1–41.8)
Weight, (%)	
Normal weight	20
Overweight	45
Obesity	29
Extreme obesity	6
Cigarette smoking status, (%)	
Never smoker	19
Ever smoker	81
Ex smoker	35
Current smoker	46
Alcohol drinking[a], (%)	
None	20
Low	52
Medium	24
High	4
Illegal drug consumption, (%)	2
Married, (%)	
Yes	89
No	11
Years working in agriculture, mean±SD	17.5±10.1
Mayan ascendancy[b], (%)	48

[a] Low: one drink (beer, brandy, tequila, ron or wine); medium: two drinks; high: three drinks.
[b] Carrying one Mayan surname.

Table 2
PON1Q192R frequencies and paraoxonase and arylesterase activities according to genotype

Genotype	Frequency	Paraoxonase activity (U/l) median (range)	*p*[a]	Arylesterase activity (U/ml) mean±SD	*p*[b]
QQ	0.21	166.2 (92.3–313.9)		108.0±13.9	
QR	0.51	461.7 (240.1–1680.5)		138.8±28.4	
RR	0.28	904.9 (369.3–1782.1)	0.0001	145.3±29.5	0.003

[a] Evaluated by ANOVA from trend on log-transformed values.
[b] Evaluated by ANOVA.

Results

Characteristics of the study population

A total of 54 eligible agricultural workers participated in the study, providing a semen and blood sample. The participants' response rate was about 26%. The demographic information of the study population is displayed in Table 1. Men had between 18–55 years old with a mean age of 39.2±10.2 years and 17.5±10.1 years working in agriculture. The BMI median value was 27.3, ranging form 21.1 to 41.8, showing a high prevalence of overweight (80%) according to the classification proposed by Flegal et al. (2004). Forty three percent of subjects were current smokers, only one participant (2%) referred drug consumption, although a high percentage of workers (80%) referred alcohol drinking; only 4% were heavy drinkers. Finally, 48% of subjects had Mayan ascendancy (at least one Mayan surname).

Safety procedures followed by the agricultural workers regarding handling pesticides

Agricultural workers did not use adequate personal protection: the use of gloves, mask or glasses was done by only 15% of participants; around 94% reported wearing a hat, 80% used water resistant boots, and 60% protected their mouth and nose. Regarding the working practices, 84% of participants reported taking a bath after a working day, 78% cleaned the used equipment and 58% of men stored pesticides in their homes.

Exposure to pesticides

Pesticide exposure in the study population was chronic (a mean value of almost 18 years working in agriculture. Farmers were

Table 3
Semen quality in the agricultural workers

Parameter	Median (range)	Abnormality (%)[a]
Morphology (%)	14 (2–41)	100
Volume (ml)	1.7 (0.5–5.5)	46
Motility (%)[b]	54.5±14.2	31
Viability (%)	82 (19–97.5)	13
Concentration ($\times 10^6$ cells/ml)[b]	92.1±50.5	6
NT-positive cells (%)	19 (5–87)	88

Reference values: morphology ≥50% normal forms; volume ≥1.5 ml; motility ≥50% motile cells; viability ≥50% live cells; concentration $\geq 20 \times 10^6$ cells/ml; NT-positive cells <10% (Sakas et al., 1996).
[a] Indicates the percentage of subjects with values outside the reference (WHO, 2002).
[b] Values are expressed as mean±SD.

Table 4
Simple and multiple linear regression analysis of semen quality and DNA integrity modeling OP exposure

Parameter	At the month of sampling			During 3 months before sampling		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	0.001	0.032	0.467	0.001	−0.039	0.750
Motility (%)	0.005	−0.0282	0.812	−0.001	−0.015	0.918
Viability (%)[c]	237.780	0.155	0.528	−10.283	0.094	0.849
Concentration (10^6 cells/ml)	0.038	0.035	0.621	0.004	0.037	0.695
Volume (ml)[d]	−0.001	0.065	0.227	−0.001	0.052	0.778
NT-positive cells (%)[d]	−0.001	−0.113	0.508	0.001	−0.104	0.772

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides at the month and during 3 months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm.

continuously exposed throughout the year to mixtures of pesticides with variable exposure frequency and type of pesticides, depending on the weather and type of crops, among other factors. Temperatures (low/high) in this part of the country are 16 °C/31 °C during December–February and 22 °C/35 °C during May–September. We did not find a statistical difference in OP exposure (either at the month of sampling or during the preceding 3 months of sampling) between summer and winter months, probably due to the wide variation that we observed in pesticide use among farmers.

Pesticides used by the agricultural workers consisted of 29 different active ingredients, including insecticides as OP [methamidophos (highly toxic), chlorpyrifos (moderately toxic), methyl parathion (extremely toxic), malathion (slightly toxic) and diazinon (moderately toxic)], carbamates [methomyl (highly toxic), carbofuran (highly toxic), carbaryl (moderately toxic), mancozeb (slightly toxic), benomyl (slightly toxic)] and pyrethroids [permethrin, cypermethrin and lambda cyhalotrin (moderately toxics)]; herbicides [paraquat, 2,4-D and glyphosate (moderately toxics)].

Regarding OP exposure indexes, a wide range was observed; however, in general, the exposure at the month of sampling (mean value of 106 arbitrary units) represented 72% of total exposure during the preceding 3 months before sampling (mean value of 146.4 arbitrary units).

PON1Q192R polymorphism and activity

PON1Q192R genotype frequencies observed in the agricultural workers are presented in Table 2. Frequencies for 192R and 192Q alleles were 0.54 and 0.46, respectively, and the frequencies for 192RR, 192QR and 192QQ genotypes were 0.28, 0.51 and 0.21, respectively. The observed PON1Q192R genotype frequencies were similar to those expected, which were in agreement with the Hardy–Weinberg equilibrium (p=0.9863). Table 2 also presents the paraoxonase and arylesterase activities as a function of PON1Q192R genotypes. The mean value observed in participants for arylesterase activity was 134.5±29.2 U/ml and the median value for paraoxonase activity was 480.1 U/l. A wide variation in PON1 activities was observed among participants and a good agreement between genotype and phenotype using paraoxon and phenylacetate as substrates. Homozygote subjects for the 192R allele had the highest levels of PON1 activity to both substrates, paraoxon (paraoxonase activity) and phenylacetate (arylesterase activity). No significant differences were observed in the general characteristics of participants and OP exposure among PON1Q192R genotypes.

Semen quality and DNA integrity of the study population

Semen quality parameters of the agricultural workers are summarized in Table 3. Overall, morphology and DNA integrity were the only parameters that showed mean values outside the reference levels (Sakkas et al., 1996; WHO, 2002). Anomalies on head shape were among the most common morphological alterations observed in spermatozoa. However, a varied percentage of participants showed abnormal values of some of the semen quality parameters; all participants had abnormal morphology, about half of farmers (46%) had low ejaculate volume, 30% showed low sperm motility and 13% of subjects had low sperm viability. Sperm concentration was the least affected parameter; only 6% of workers had low concentration (less than 20×10^6 cells /ml). Alteration on sperm DNA integrity, evaluated by the percentage of NT-positive cells, showed a range of 5–87%, and about 88% of workers showed higher values than that reported in fertile men (<10%, Sakkas et al., 1996). Individuals living in the same area but not occupationally exposed to pesticides showed a mean value of NT-positive cells of 6.3% (data not shown). Finally, no association was observed between sperm DNA integrity and semen quality parameters, but there was a borderline significance (p=0.087) with sperm viability (data not shown).

Effect on semen quality and DNA integrity by organophosphorous pesticide exposure

Table 4 shows the multiple linear regression analysis between OP exposure and parameters of semen quality and DNA integrity. Regarding semen quality, we found a lack of association between conventional semen quality parameters (sperm morphology, viability, motility and concentration, ejaculate volume) and OP exposure, either at the month of collecting the samples or during the preceding 3 months before sampling. On the other hand, the percentage of NT-positive cells was neither associated with OP exposure considering

Table 5
Multiple linear regression modeling OP exposure at the month of sampling according to PON1Q192R genotype

Genotype									
Parameter	QQ			QR			RR		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	0.001	−0.016	0.951	0.001	−0.1335	0.650	−0.029	−0.0026	0.372
Motility (%)	−0.140	0.870	0.214	0.052	−0.022	0.121	−0.140	0.870	0.214
Viability (%)[c]	−2143.942	0.626	0.270	224.370	0.0308	0.709	−13,106.55	0.1755	0.086
Concentration (10^6 cells/ml)	−0.695	0.709	0.102	0.131	0.009	0.149	0.882	0.0332	0.547
Volume (ml)[d]	0.018	0.848	0.003	−0.001	0.023	0.534	−0.014	−0.075	0.384
NT-positive cells (%)[d]	−0.003	−0.129	0.448	−0.001	−0.072	0.598	0.013	0.279	0.025

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides 3 months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm.

Table 6
Multiple linear regression modeling OP exposure during 3 months before sampling according to PON1Q192R genotypes

Genotype									
Parameter	QQ			QR			RR		
	Coefficient	r^2	p^a	Coefficient	r^2	p^a	Coefficient	r^2	p^a
Morphology (%)[b]	−0.001	−0.064	0.878	−0.001	−0.258	0.585	−0.003	0.148	0.098
Motility (%)	−0.018	0.845	0.434	0.019	0.204	0.158	−0.053	0.385	0.043
Viability (%)[c]	−425.410	0.625	0.313	−126.286	−0.049	0.624	−1361.658	0.419	0.008
Concentration (10^6 cells/ml)	−0.114	0.797	0.115	0.051	0.004	0.191	−0.141	0.265	0.102
Volume (ml)[d]	0.003	0.909	0.001	−0.001	0.035	0.640	−0.001	−0.064	0.358
NT-positive cells (%)[d]	−0.001	−0.901	0.696	−0.006	−0.114	0.366	0.003	0.202	0.059

[a] Adjusted by cigarette smoking, alcohol drinking, caffeine intake and exposure to all pesticides during 3 months before collecting the samples.
[b] Values are transformed by square root.
[c] Values are transformed by cubic root.
[d] Values are transformed by logarithm.

the month of collecting the samples nor with the exposure during 3 months before sampling.

Effect of organophosphorous pesticide exposure on semen quality and DNA integrity according to PON1Q192R genotype

We proceeded to explore the role of PON1Q192R genetic polymorphism on the relationship between OP exposure and parameters of semen quality and DNA integrity. When subjects were separated by their genotype, the multiple linear regression analysis showed a lack of association between OP exposure at the month of collecting the samples and semen quality parameters, but there was a borderline significance ($p=0.086$) with sperm viability only in homozygote subjects for the 192R allele (Table 5); however, interestingly, dose–effect relationships ($p<0.05$) were observed between sperm motility and viability and OP exposure during 3 months before sampling only in subjects featuring the 192RR genotype, and homozygote subjects for the 192R allele also had a slight decrease in sperm morphology ($p=0.098$) associated with OP exposure (Table 6). These data suggest that cells at any stage of spermatogenesis are a target of OP toxicity in susceptible individuals (192RR genotype). A significant positive association between OP exposure indexes and ejaculate volume was observed; however, we do not have a plausible explanation for this finding.

Regarding the effect on DNA integrity, homozygote subjects for the 192R allele showed dose–effect relationships with OP exposure at the month of sampling ($p=0.025$) (Table 5) and during 3 months before sampling ($p=0.059$) (Table 6). This suggests, at least, that cells at the last steps of spermatogenesis are susceptible to DNA damage by OP exposure. Also, our results clearly indicate that the adverse effects on semen quality and DNA integrity caused by OP exposure are modulated by PON1Q192R polymorphism; workers featuring the 192RR genotype are more susceptible to OP adverse effects than subjects carrying the Q allele.

On the other hand, paraoxonase activity showed an inverse association only with sperm concentration, and arylesterase activity was not associated with any semen quality parameter (data not shown).

Discussion

The present study evaluated the relationship between PON1Q192R polymorphism and the effects of OP exposure on semen quality and sperm DNA integrity in traditional farmers from southeastern Mexico. The major findings of this study were that the effects on sperm DNA integrity and semen quality parameters (viability, motility and morphology) were modified by PON1Q192R polymorphism, showing that subjects featuring the 192RR genotype were more susceptible to develop adverse effects by the exposure to OP mixture used by our population. Additionally, we also suggest that cells at the last stages of their maturation and probably at earlier stages of spermatogenesis are sensitive to OP toxicity.

Epidemiological studies have shown that semen quality is decreased by occupational and environmental exposures to pesticides, including OP, regardless of the interaction with OP metabolizing enzyme polymorphisms. Poor semen quality has been reported in workers from a manufacturing OP factory (ethyl and methyl parathion and methamidophos) (Padungtod et al., 1999), in men environmentally exposed to diazinon (Swan et al., 2003), in agricultural workers exposed to a mixture of OP (methyl parathion, methamidophos, dimethoate and diazinon, among others) (Sánchez-Peña et al., 2004), in farmers exposed to fenitrothion, dichlorvos, chlorpyrifos, diazinon and propetamphos (Kamijima et al., 2004), and in men environmentally exposed to chlorpyrifos (Meeker et al., 2004a).

The role of genetic polymorphisms as determinants of health is explored in many areas of public health research, including epidemiological and toxicological studies (Kelada et al., 2003). The evidence of the wide variation in serum PON1 activity among individuals (La Du et al., 1986) raised the hypothesis that subjects with low PON1 activity might be more susceptible to the toxicity of some OP, such as methyl parathion and chlorpyrifos that are hydrolyzed faster by the 192R alloenzyme. Epidemiological studies have explored the role of PON1Q192R genotype or phenotype on the susceptibility to OP exposure to develop neurological toxicity and health problems (Cherry et al., 2002; Haley et al., 1999; Hotopf et al., 2003; Lee et al., 2003; Mackness et al., 2000), and DNA damage in somatic cells (Liu et al., 2006; Lee et al., 2007). However, there is only one study on germinal cells, where authors did not observe a gene-dose effect of PON1Q192R genotype with respect to male reproductive toxicity in a case-control study in Chinese pesticide factory workers (Padungtod et al., 1999). Our results suggest an interaction between PON1Q192R polymorphism and OP exposure in relation to sperm DNA integrity and semen quality in agricultural workers exposed to OP mixtures; workers featuring the 192RR genotype were more susceptible to OP toxicity than subjects featuring the 192QR or 192QQ genotypes. Considering that homozygote 192RR subjects represent 28% of the studied population, their potential risk to developing adverse reproductive effects deserves to be taken into consideration and further studies seem worthy to elucidate the role of PON1 polymorphisms in OP toxicity in other populations.

There are some reports that failed in observing a role of PON1 polymorphism on pesticide-related toxicity. Liu et al. (2006) reported no significant association between PON1 genotype and DNA damage (Comet assay) in Taiwanese fruit growers, suggesting that it may be due to the small number of subjects exposed at a substantial level (high pesticide exposure). Lee et al. (2007) did not find a consistent trend suggestive of the role of PON1 polymorphism in the generation of DNA oxidative damage (levels of 8-OHdG) in 18 indoor sprayers, and similarly, authors suggest that their results need to be confirmed in a larger population. Finally, Padungtod et al. (1999) in a case-control study in Chinese OP manufacturing workers did not show an interaction between PON1Q192R genotype and OP pesticides exposure on male

reproductive outcomes, suggesting that it may be due to the small sample size ($n=32$ exposed workers). Although a large sample size may be required for detection of gene–environment interactions, positive results observed in our study ruled out bias with regard to genotype, OP exposure or reproductive effects.

In this study ($n=54$), to further evaluate the interaction between PON1Q192R genotype and OP exposure in relation to sperm DNA integrity and semen quality, we evaluated the effects considering the exposure at two different times: at the month of sampling, representing the exposure to last step of spermatogenesis and epididymal maturation, and during the preceding 3 months before sampling, to ensure the exposure to cells at all stages of their maturation. The strategy of evaluating the effects on spermatozoa with a time frame between the exposure and semen analysis permits the evaluation of the toxic effects on spermatogenic cells at different stages of maturation, because cells at each stage have different susceptibilities to chemical damage (Marchetti et al., 2001). Since the spermatogenic process in men lasts 2.5 months (74 days), one month before the analysis represents the exposure to cells at spermiogenesis (spermatids and spermatozoa) and epididymal maturation, while during 3 months before the analysis represents the exposure to cells during a complete spermatogenic cycle (Johnson, 1997). With our data we can strongly suggest that PON1Q192R polymorphism modulates the effects of occupational OP exposure on sperm DNA integrity at least during last steps of spermatogenesis, when cells are spermatids and spermatozoa or at their transit through the epididymis. This is supported by the association observed between sperm DNA damage and the exposure at the month of sampling in homozygote subjects for the 192R allele. The associations observed between OP exposure during 3 months before sampling and sperm DNA damage and semen quality denote that spermatogenic cells may be target of OP toxicity at any stage of their maturation. Further studies will be necessary to elucidate the specific stage at which spermatogenic cells are more sensitive to OP exposure.

Previous data from our group have suggested that cells that are at meiosis as well as at late spermatids and epididymal maturation at the time of exposure are target of methyl parathion and diazinon toxicity (Piña-Guzmán et al., 2005, 2006). Similarly, Burruel et al. (2000) suggested that methamidophos might promote the phosphorylation of components of the mitotic spindle and thus contribute to sperm DNA damage, and Sega (1991) reported that late spermatids and early spermatozoa are sensitive to alkylating agents. These two unique events of spermatogenesis, meiosis (spermatocytes) and chromatin condensation (including late spermatids and maturing spermatozoa, when histones are replaced by protamines and disulfide bond formation occurs, respectively) are at high risk of genetic damage. Meiosis is a long-lasting stage where important processes for genetic continuity occur (Adler, 1982), and chromatin condensation is due to the lack or reduced mechanisms of defense such as DNA-repair capacity (Sotomayor and Sega, 2000) and anti-oxidant enzymes (Aitken and Fisher, 1994). On the other hand, Mathew et al. (1992) showed a high percentage of abnormal spermatozoa by methyl parathion when cells were treated as spermatocytes in mouse, and Sobarzo and Bustos-Obregón (2000) injected ethyl parathion to 7 days old mice (when spematogenic cells were immature) and observed sperm head and flagellum anomalies and chromatin alterations in cells collected from the epididymis 28 and 50 days after the exposure. Furthermore, Bustos-Obregón and González-Hormazabal (2003) suggested that the increased percentage of teratozoospermia by a single dose of malathion may result from genetic damage caused to spermatogonias.

Limited information is available about OP genotoxicity to male germinal cells in humans. OP have been associated with sperm chromosomal damage expressed as increased frequency of aneuploidy (X, Y, and 18 chromosomes) in Mexican agricultural workers exposed to a mixture of OP (Recio et al., 2001), and in Chinese workers from a OP manufacturing plant (Padungtod et al., 1999). In addition, environmental exposure to chlorpyrifos and occupational exposure to a mixture of OP have been associated with increased human sperm DNA fragmentation (Meeker et al., 2004b; Sánchez-Peña et al., 2004). The dose–effect relationships between both OP exposure indexes and DNA damage observed in this study demonstrate that OP are genotoxic agents to male germ cells. The *in situ*-nick translation detects the incorporation of bases into nicks in spermatozoa using the anti-sense strand as template and DNA polymerase I. Studies conducted in humans suggest that normal males present endogenous nicks in <10% of their spermatozoa (Sakkas et al., 1996) and values of NT-positive spermatozoa higher than 10% are more frequent in those couples with problems in establishing a pregnancy (Tomlinson et al., 2001). Furthermore, the presence of sperm DNA damage in mature spermatozoa raises the concern about delivering an altered male genome to the developing embryo (Hales et al., 2005). In this regard, Burruel et al. (2000) reported that single doses of the OP insecticide methamidophos caused a decrease in embryo cell number accompanied by an increase in the proportion of degenerating embryos at post exposure week 4.

The lack association observed between sperm DNA damage and conventional semen quality parameters (except for viability) is in agreement with other studies (Sánchez-Peña et al., 2004; Spano et al., 1998; Xia et al., 2005), supporting the notion that sperm chromatin or DNA integrity are independent parameters of semen quality and may show early stages of damage.

We considered as an exposure measure the indexes created from the exposure history reported by participants, similar to other groups (Liu et al., 2006; Tahmaz et al., 2003); therefore, the interaction observed between OP exposure (at both times) and PON1Q192R genotype in regard to sperm DNA and semen quality relies on a exposure ranking rather than absolute levels of pesticide exposure. Quantification of OP main metabolites, dialkylphosphates (DAP) in urine has been traditionally used as OP exposure indicator, but because DAP are rapidly excreted, 80–90% within 48 h (Vasilic et al., 1993), a single urine sample may not be representative for a longer-term exposure. In addition, studies have shown no differences between levels of DAP before or during the pesticide use season (Recio et al., 2001) and previously our group did not observe association between DAP and semen quality in farmers of north Mexico (Sánchez-Peña et al., 2004). We evaluated the exposure by indexes of use of all pesticides and the group of OP by questionnaire information similar to other studies.

Regarding PON1Q192R allele frequencies observed in this half breed population from southern Mexico (0.54R and 0.46Q), they were similar to those previously reported in other Mexican populations (Gamboa et al., 2006; Rojas-García et al., 2005), and Afro-Brazilian, Caribbean, and Japanese populations (Allebrant et al., 2002; Chen et al., 2003; Suehiro et al., 2000), but different from Spaniards and Caucasians (Chen et al., 2003; Hernández et al., 2003). However, the high percentage (28%) of homozygote subjects with the 192RR genotype makes this population highly susceptible to develop reproductive effects due to their continuous exposure to OP. On the other hand, a wide variation in PON1 activity (phenotype) was observed, in agreement with other studies (Brophy et al., 2001; Chen et al., 2003; Rojas-García et al., 2005), but it did not show an association with OP exposure effects on sperm DNA integrity or semen quality. This study is the first one on reporting PON1Q192R genotype frequencies in a Mayan population.

The knowledge that PON1 is a HDL-associated enzyme that prevents lipid peroxide accumulation (Mackness et al., 1991), that HDL isolated from subjects featuring the 192RR genotype retains <1% of the anti-oxidant function compared to >50% in subjects with the 192QQ genotype (Mackness et al., 1998), and that OP exposure induces oxidative stress as a mechanism of their toxicity in humans and animals (Ranjbar et al., 2002; Piña-Guzmán et al., 2006; Ref43Quitanilla-Vega et al., in press), supports our finding that farmers featuring the 192RR genotype showed more susceptibility to reproductive effects by OP exposure. Participants of this study were exposed to a

mixture of pesticides, mostly OP: methamidophos, chlorpyrifos, methyl parathion, malathion and diazinon in that order. PON1 activity towards several OP is substrate-dependent: the 192R alloenzyme hydrolyzes paraoxon and chlorpyrifos oxon faster than the 192Q alloenzyme, whereas diazoxon is hydrolyzed faster by the 192Q alloenzyme (Davies et al., 1996). PON1 activity towards methamidophos (the OP mostly used by our population) is not known, but we can suggest that it is hydrolyzed by the 192Q alloenzyme, since homozygote 192RR subjects showed more susceptibility to OP reproductive toxicity. Studies are in progress to elucidate the PON1 alloenzyme that hydrolyzes methamidophos.

We are aware of some inherent limitations of our study. First, the low participation rate may have introduced selection bias in the risk estimates, since those workers more affected by pesticide exposure might be more willing to provide semen samples. Therefore, the participating farmers may not truly represent the whole population. Second, although estimating the exposure through questionnaire, no other measurement of chronic OP exposure is available.

In summary, our data show that occupational OP exposure was associated with adverse effects on sperm DNA integrity and semen quality only among susceptible workers; those who possess the homozygous 192R alleles. Thus, we demonstrated for the first time, the susceptible PON1Q192R genotype for OP exposure affecting male reproduction, and highlighted that more work is needed in this field of study to determine the role of PON1 genetic polymorphisms on the susceptibility to different OP reproductive toxic effects.

Acknowledgments

The authors gratefully acknowledge the assistance of authorities from Muna, Yucatán and personnel from the Schools of Medicine and Chemistry from the University of Yucatán during the survey, M.S. Rosa Leticia González Navarrete and Jorge Castro Mañé for collaborating on the survey, Carlos May-Pech for helping on phenotyping, and Gerardo Martínez Aguilar for his help in data base capture. This work was supported by CONACYT-Mexico (Grant CO1-134). NPH was a recipient of a scholarship from CONACYT-Mexico.

References

Aitken, R.J., Fisher, H., 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioassays 16, 259–267.
Adler, I.D., 1982. Mouse spermatogonia and spermatocyte sensitivity to chemical mutagens. Cytogenet. Cell Genet., 33, 95–100.
Allebrant, K.V., Souza, R.L., Chautard-Freire-Maia, E.A., 2002. Variability of the paraoxonase gene (PON1) in Euro- and Afri-Brazilians. Toxicol. Appl. Pharmacol. 3, 151–156.
Brophy, V.H., Jampsa, R.L., Clendenning, J.B., Mckinstry, L.A., Furlong, C.E., 2001. Effects of 5′regulatory-region polymorphism on paraoxonase-gene (PON1) expression. Am. J. Hum. Genet. 68, 1428–1436.
Burruel, V.R., Raabe, O.G., Overstreet, J.W., Wilson, B.W., Wiley, L.M., 2000. Paternal effects from methamidophos administration in mice. Toxicol. Appl. Pharmacol. 165, 148–157.
Bustos-Obregon, E., González-Hormazabal, P., 2003. Effect of a single dose of malathion on spermatogenesis in mice. Asian J. Androl. 5, 105–107.
Chen, J., Kumar, M., Chan, W., Berkowitz, G., Wetmur, J.G., 2003. Increased of genetic variation on PON1 activity in neonates. Environ. Health Perspect. 111, 1403–1409.
Cherry, N., Mackness, N., Durrington, P., Povey, A., Dippnall, M., Smith, T., Mackness, B., 2002. Paraoxonase (PON1) polymorphisms in farmers attributing ill health to sheep dip. Lancet. 359, 763–764.
Davies, H.G., Richter, R.J., Keifer, M., Broomfield, C.A., Sowalla, J., Furlong, C.E., 1996. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat. Genet. 14, 334–336.
Eckerson, H.W., White, C.M., La Du, B.N., 1983. The human serum paraoxonase/arylesterase polymorphism. Am. J. Hum. Genet. 35, 1126–1138.
Flegal, K.M., Ogden, C.L., Carrol, M.D., 2004. Prevalence and trends in overweight in Mexican–American adults and children. Nutr. Rev. 62, 144–148.
Furlong, C.E., Richter, R.J., Seidel, S.L., Motulsky, A.G., 1988. Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon. Am. J. Hum. Genet. 43, 230–238.
Gamboa, R., Zamora, J., Rodríguez-Pérez, J.M., Fragoso, J.M., Cardoso, G., Posadas-Romero, C., Vargas-Alarcón, G., 2006. Distribution of paraoxonase PON1 gene polymorphisms in Mexican populations Its role in the lipid profile. Exp. Mol. Pathol. 80, 85–90.
Geldmacher-von Mallinckrodt, M., Diepgen, T.L., 1988. The human serum paraoxonase-polymorphism and specificity. Toxicol. Environ. Chem. 18, 79–196.
Hales, B.F., Aguilar-Mahecha, A., Robaire, B., 2005. The stress response in gametes and embryos alter paternal chemical exposures. Toxicol. Appl. Pharmacol. 207, 514–520.
Haley, R.W., Billecke, S., La Du, 1999. Association of low PON1 type Q (type A) arylesterase activity with neurologic symptom complexes in Gulf War veterans. Toxicol. Appl. Pharmacol. 157, 227–233.
Hernández, A.F., Mackness, B., Rodrigo, L., López, O., Pla, A., Gil, F., Durrington, P.N., Pena, G., Parrón, T., Serrano, J.L., Mackness, M.I., 2003. Paraoxonase activity and genetic polymorphisms in greenhouse workers with long term pesticide exposure. Hum. Exp. Toxicol. 22, 565–574.
Hotopf, M., Mackness, M.I., Nikolau, V., Collier, D.A., Curtis, C., David, A., Durrington, P., Hull, L., Ismail, K., Peakman, M., Unwin, C., Wessely, S., Mackness, B., 2003. Paraoxonase in Persian Gulf War Veterans. J. Occup. Environ. Med. 45, 668–675.
IARC, 1991. IARC monographs on the evaluation of carcinogenic risks to humans. Occupational exposures in insecticide application, and some pesticide, vol. 53. IARC, Lyon, France.
INEGI, 2005. Instituto Nacional de Estadística Geografía e Informática. Available at: http://www.inegi.gob.mx.
Johnson, L., 1997. Efficiency of spermatogenesis in humans and animals. College Station, Texas.
Jokanovick, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166, 139–160.
Jørgensen, N., Andersen, A.G., Eustache, F., Irvine, D.S., Suominen, J., Petersen, J.H., Andersen, A.N., Auger, J., Cawood, E.H.H., Horte, A., Jensen, T.K., Jouannet, P., Keiding, N., Vierula, M., Toppari, J., Skakkebæk, N.E., 2001. Regional differences in semen quality in Europe. Hum. Reprod. 16, 1012–1019.
Kamijima, M., Hibi, H., Gotoh, M., Taki, K., Saito, I., Wang, H., Itohara, S., Yamada, T., Ichihara, G., Shibata, E., Nakajima, T., Takeuchi, Y., 2004. A survey of semen indices in insecticide sprayers. J. Occup. Health. 46, 109–118.
Kelada, S.N., Eaton, D.L., Wang, S.S., Rothman, N.R., Khoury, M., 2003. The role of genetic polymorphisms in environmental health. Environ. Health Perspect. 111, 1055–1064.
La Du, B.N., Piko, J.I., Eckerson, H.W., Vincent-Viry, M., Siest, G., 1986. An improved method for phenotyping individuals for the human serum paraoxonase arylesterase polymorphism. Ann. Boil. Clin. 44, 369–372.
La Du, B.N., 1996. Structural and functional diversity of paraoxonases. Nat. med. 2, 1186–1187.
Lee, B.W., London, L., Paulauskis, J., Myers, J., Christiani, D.C., 2003. Association between human paraoxonase gene polymorphism and chronic symptoms in pesticide-exposed workers. Occup. Environ. Med. 45, 118–122.
Lee, C.H., Kamijima, M., Kim, H., Shibate, E., Ueyama, J., Suzuki, T., Takagi, K., Saito, I., Gotoh, M., Hibi, H., Naito, H., Nakajima, T., 2007. 8-Hydroxydeoxyguanosine levels in human leukocyte and urine according to exposure to organophosphorus pesticides and *paraoxonase* 1 genotype. Int. Arch. Occup. Environ. Health 80, 217–227.
Li, H.L., Liu, D.P., Liang, C.C., 2003. Paraoxonase gene polymorphisms, oxidative stress, and diseases. J. Mol. Med. 81, 766–779.
Liu, Y.J., Huang, P.L., Chang, Y.F., Chen, Y.H., Chiou, Y.H., Xu, Z.L., Wong, R.H., 2006. *GSTP1* genetic polymorphism is associated with a higher risk of DNA damage in pesticide-exposed fruit growers. Cancer Epidemiol. Biomarkers Prev. 15, 659–666.
Mackness, M.I., Arrol, S., Durrington, P.N., 1991. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. FEBS Lett. 286, 152–154.
Mackness, B., Mackness, M.I., Arrol, S., Turke, W., Durrington, P.N., 1998. Effect of the human serum paraoxonase 55 and 192 genetic polymorphism on the protection by high density lipoprotein against low density lipoprotein oxidative modification. FEBS Lett. 423, 57–60.
Mackness, B., Durrington, P.N., Mackness, M.I., 2000. Low paraoxonase in Persian Gulf War veterans self-reporting Gulf War Syndrome. Biochem. Biophys. Res. Commun. 276, 729–733.
Marchetti, F., Bishop, J.B., Lowe, X., Generoso, W.M., Hozier, J., Wyrobek, A.K., 2001. Etoposide induces hereditable chromosomal aberrations and aneuploidy during male meiosis in the mouse. Proc. Natl. Acad. Sci. 98, 3952–3957.
Mathew, G., Vijayalaxmi, K.K., Rahiman, M.A., 1992. Methyl parathion-induced sperm shape abnormalities in mouse. Mutat. Res. 280, 169–173.
Meeker, J.D., Ryan, L., Barr, D.B., Herrick, R.F., Bennertt, D.H., Bravo, R., Hauser, R., 2004a. The relationship of urinary metabolites of carbaryl/naphthalene and chlorpyrifos with human semen quality. Environ. Health Perspect. 112, 1665–1670.
Meeker, J.D., Singh, N.P., Ryan, L., Duty, L.R., Barr, D.B., Herrick, R.F., Bennett, H.D., Hausser, Russ, 2004b. Urinary levels of insecticide metabolites and DNA damage in human sperm. Human. Reprod. 19, 2573–2580.
Padungtod, C., Niu, T., Wang, Z., Savitz, D.A., Christiani, C., Ryan, L.M., Xu, X., 1999. Paraoxonase polymorphism and its effect on male reproductive outcomes among Chinese pesticide factory workers. Am. J. Ind. Med. 36, 379–387.
Padungtod, C., Savitz, D.A., Overstreet, J.W., Christiani, D.C., Ryan, L.M., Xu, X., 2000. Occupational pesticide exposure and semen quality among Chinese workers. J. Occup. Environ. Med. 42, 982–992.
Piña-Guzmán, B., Solís-Heredia, Quintanilla-Vega, B., 2005. Diazinon alters sperm chromatin structure in mice by phosphorilating nuclear protamines. Toxicol. Appl. Pharmacol. 202, 189–198.
Piña-Guzmán, B., Solís-Heredia, M.J., Rojas-García, A.E., Urióstegui-Acosta, M., Quintanilla-Vega, B., 2006. Genetic damage caused by methyl-parathion in mouse spermatozoa is related to oxidative stress. Toxicol. Appl. Pharmacol. 216, 216–224.
Primo-Parmo, S.L., Sorenson, R.E., Teiber, J., La Du, B.N., 1996. The human serum paraoxonase/arylesterase gene (PON1) is a member of a multigene family. Genomics. 33, 498–509.
Quitanilla-Vega, B., Pérez-Herrera, N., Piña-Guzmán, B., in press. Organophosphorous pesticides alter sperm chromatin structure and DNA integrity: mechanisms of reproductive toxicity. Environ. Res. J.

Ranjbar, A., Pasalar, P., Abdollahi, M., 2002. Induction of oxidative stress and acetylcholinesterase inhibition in organophosphorous pesticide manufacturing workers. Hum. Exp. Toxicol. 21, 179–182.

Recio, R., Robbins, W.A., Ocampo-Gómez, G., Borja-Aburto, V., Morán-Martínez, J., Froines, J.R., García Hernández, Rosa Ma., Cebrián , M.E., 2001. Organophosphorous pesticide exposure increases the frequency of sperm sex null aneuploidy. Environ. Health Perspect. 109, 31–34.

Rojas-García, A.E., Solís-Heredia, M.J., Piña-Guzmán, B., Vega, L., López-Carrillo, L., Quintanilla-Vega, B., 2005. Genetic polymorphism and activity of PON1 in a Mexican population. Toxicol. Appl. Pharmacol. 205, 282–289.

Sakkas, D., Urner, F., Bianchi, P.G., Bizzarro, D., Wagner, I., Jaquenoud, N., Manicardi, G., Campana, A., 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection (ICSI). Hum. Reprod. 11, 843–973.

Sánchez-Peña, L.C., Reyes, B.E., López-Carrillo, L., Recio, R., Morán-Martínez, J., Cebrián, M.E., Quintanilla-Vega, B., 2004. Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol. Appl. Pharmacol. 196, 108–113.

Sega, G.A., 1991. Adducts in sperm protamine and DNA vs. mutation frequency. Prog. Clin. Biol. Res. 372, 521–530.

Sobarzo, C., Bustos-Obregón, E., 2000. Sperm quality in mice acutely treated with parathion. Asian J. Androl. 2, 147–150.

Sotomayor, R.E., Sega, G.A., 2000. Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update. Environ. Mol. Mutagen. 36, 255–265.

Spano, M., Kolstad, H., Larsen, S.B., Cordelli, E., Leter, G., Giwercman, A., 1998. The applicability of the flow cytometric sperm chromatin structure assay to epidemiological studies. Hum. Reprod., 13, 2495–2505.

Suehiro, T., Nakamura, T., Inoue, M., Shinoki, T., Ikeda, Y., Kumon, Y., Shindo, M., Tanaka, H., Hashimoto, K., 2000. A polymorphism upstream from the human paraoxonase (PON1) gene and its association with PON1 expression. Atherosclerosis 150, 295–298.

Summer, A.T., Taggart, M.H., Mezzanotte, R., Ferruci, L., 1990. Patterns of digestion of human chromosomes by restriction endonucleases demonstrated by in situ nick translation. Histochem. J. 22, 639–652.

Swan, S.H., Kruse, R.L., Liu, F., Barr, D.B., Drobnis, E.Z., Redmon, J.B., Wang, C., Brazil, C., Overstreet, J.W., the Study for Future Families Research Group, 2003. Semen quality in relation to biomarkers of pesticide exposure. Environ. Health Perspect. 111, 1478–1484.

Tahmaz, N., Soutar, A., Cherrie, J.W., 2003. Chronic fatigue and organophosphate pesticides in sheep farming: a retrospective study amongst people reporting to a UK pharmacovigilance scheme. Ann. Occup. Hyg. 47, 261–267.

Tomlinson, M.J., Moffatt, O., Manicardi, G.C., Bizarro, D., Afnan, M., Sakkas, D., 2001. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum. Reprod. 16, 2160–2165.

Vasilic, Z., Drevenkar, V., Stengl, B., Frobe, Z., Rumenjak, V., 1993. Diethylphosphorous metabolites in serum and urine of persons poisoned by phosalone. Chem. Biol. Interact. 87, 305–313.

World Health Organization, 2002. WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction. Cambridge Univ. Press, Cambridge.

Xia, Y., Cheng, S., Bian, Q., Xu, Lichun, Collins, M.D., Chang, H.C., Song, Li, Liu, J., Wang, S., Wang, X., 2005. Genotoxic effects on spermatozoa of carbaryl-exposed workers. Toxicol. Sci. 85, 615–623.