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Chronic exposure to organophosphate pesticides as an important challenge in promoting reproductive health: A comparative study

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Abstract:

INTRODUCTION: Organophosphate compounds (OPCs) are suspected to make changes in reproductive function by oxidant/antioxidant balance disruption in the brain, consequently impairing hypothalamic, pituitary endocrine functions, and gonadal processes. The aim of this study was to investigate the effects of occupational exposure to OPCs on the reproductive system of farm workers, in Hamadan, Iran.

MATERIALS AND METHODS: A comparative study was conducted in rural farmers and urban men aged 20–40 years. After sampling and analysis of semen quality parameters (such as sperm count, sperm motility, progressive sperm motility, and sperm morphology), serum butyrylcholinesterase (BChE) activity (a specific biomarker in OPCs exposure), as well as total antioxidant capacity, nitric oxide, and lipid peroxidation levels for both semen and serum samples were determined. In addition, serum samples were analyzed for reproductive hormones, including follicle-stimulating hormone, luteinizing hormone (LH), and testosterone.

Results: Our findings showed that the number of sperms ($P = 0.04$), their motility ($P < 0.001$), and progressive status ($P < 0.001$) in rural farmers were significantly lower than the urban population. In addition, a significant decrease was observed in BChE activity ($P < 0.001$) and LH level ($P < 0.001$), and also a remarkable increase was found in testosterone level ($P = 0.0014$) in the serum of rural farmers compared to the urban population. Along with a decrease in semen total antioxidant capacity, a positive significant correlation was found between sperm motility and semen antioxidant capacity ($r = 0.45$; $P < 0.05$).

Conclusion: Exposure to OPCs may affect reproductive outcomes through impairing hypothalamic and/or pituitary endocrine dysfunctions and gonadal processes in farmers.

Keywords:

Farmers, infertility, organophosphorus compounds, oxidative stress, sperm quality

Introduction

Pesticides are used extensively in tropical agriculture to increase the crop yield. Application of these agents is expected to increase based on increases in the world population and the need for more food

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supply. It is predicted that, between 1995 and 2020, the use of pesticides will increase by at least two to three times.^[1] Among these, organophosphorus compounds (OPCs) are potent neurotoxic chemicals that are characterised by their inhibitory action on acetylcholinesterase (AChE) enzyme activity in the cholinergic nerve terminal.^[2]

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Acute exposures to OPCs lead to the overstimulation of the nicotinic and muscarinic receptors, and often result in death if not managed properly.^[3] In addition, there are other derived effects from chronic exposure such as carcinogenesis, immunotoxicity, neurobehavioral hazards, endocrine alterations, or adverse effects on the human reproductive system, which have not yet been fully characterized.^[4]

Some epidemiological studies indicate that pesticides may be associated with infertility in male. For instance, Neghab *et al.* reported that the prevalence of primary infertility was remarkably higher among farm workers than in the normal population, in the Kavar region of Fars province, Southern Iran.^[5] Furthermore, alterations in serum reproductive hormone, such as testosterone, have also been described in men exposed to OPCs.^[6,7] Panuwet *et al.* conducted a cross-sectional study in male Thai farmers and reported a positive relationship between some OPCs pesticides and total testosterone level.^[8] In other study, Jamal *et al.* reported a tendency for increased LH and follicle-stimulating hormone (FSH) levels in pesticide sprayers.^[9] These changes could be associated with semen quality in infertile men, as noted by Mehrpour *et al.*^[10] The physiological levels of reactive oxygen species (ROS) and nitric oxide (NO) are important for cell signaling processes and cell function in different types of cells such as sperm. For instance, ROS can promote the acrosome reaction, whereas the presence of superoxide dismutase and catalase inhibits this reaction. The pathway of inducing the acrosome reaction seems to be ROS-modulated tyrosine phosphorylation. Subsequently, tyrosine phosphorylation enhances sperm membrane binding to the zona pellucida glycoprotein 3, promoting sperm-oocyte fusion.^[11] In addition, it has been shown that NO affects sperm motility, acts as chemoattractant, and modulates the acrosomal reaction.^[12,13]

However, when ROS/NO exceeds the cellular antioxidant capacity, it induces oxidative/nitrosative stress.^[14] This process could be one of the causes of infertility in men that are chronically exposed to OPCs. Karafs (35°21'48"N 49°17'56"E) is a rural area in Hamadan Province, Iran, where most of the people work as farmers. In this region, OPCs are one of the most important pesticides used in agriculture.

Considering the numerous cases of infertility in this rural population, the present study was designed to examine the semen quality and serum reproductive hormone profiles of the farmers in this region and to evaluate the oxidative stress biomarkers in the serum and semen collected from this population.

Materials and Methods

All chemicals were obtained from Merck, Darmstadt, Germany, unless otherwise stated. 5,5'-Dithiobis

(2-nitrobenzoic acid) (DTNB), N-(1-naphthyl) ethylenediamine dihydrochloride (NED), 2-thiobarbituric acid (TBA), and 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Study design and population

A comparative study was conducted between 2014 and 2016 with a random sampling of men aged 20–40 years from the people of the east of Hamadan, Iran. After statistical studies, the control group was selected from the men of the urban region, and the case group was selected from the farmers of the rural region in the Hamadan Province (Karafs region), Iran. Participants that previously had a vasectomy, cancer, endocrine, or reproductive disorders were excluded from the study. In addition, urban residents with the history of farmer or pesticide exposure were also excluded from the control group. Finally, 30 urban men (control) and 30 men rural farmers (case) participated in the study. It should be noted that the study protocol was approved by the Hamadan University of Medical Science Ethics Committee (No. 9307086068), and all participants read and signed an informed consent form.

Semen and blood collection

An intravenous blood sample was collected in heparin tube between 9:00 and 10:30 in the morning after 12 h overnight fast. Serum was separated from the whole blood by centrifugation at 3000 g for 5 min. Specimens were stored at –20°C under suitable conditions until analysis. The semen collection was done according to the recommendations of the fifth edition of the Manual for the analysis and processing of human semen.^[15] The participating individuals had been asked to abstain from ejaculation for at least 48 h before semen collection. It should be noted that ejaculation abstinence time was noted as the time between current and previous ejaculation as described by the participating individuals. Semen specimens were kept in an incubator at 37°C for 20 min before analysis.

Serum biochemical analysis

Butyrylcholinesterase (BChE), as indexes of OPCs exposure, was assayed in serum samples according to the reaction of thiocholine with DTNB that its color change was detected at 412 nm using a microplate reader (Synergy HTX, Biotek, USA).^[1] Serum levels of luteinizing hormone (LH) and FSH and total testosterone were analyzed by enzyme-linked immunoassay technique according to the kit brochure (Pishtaz Teb Company, Iran).

Semen analysis

In addition to semen macroscopic parameters such as pH and volume (ml), microscopic indexes including sperm

count (million/ml), sperm motility (%), progressive sperm motility (%), and sperm morphology were analyzed using CASA system (Hamilton-Thorn Version 10, HTM-IVOS).^[15]

Evaluation of oxidant/antioxidant status

The seminal plasma and blood serum were used for oxidative stress experiments. TBA-reactive substances method was used for lipid peroxidation (LPO) assay as described by Nili-Ahmadabadi *et al.*^[11] Briefly, seminal plasma and/or serum samples were mixed with TBA (0.2%) in H₂SO₄ (0.05 M) and heated for 30 min in boiling water bath. By-products of LPO were extracted by n-butanol, and absorbance was determined at 532 nm. Malondialdehyde (MDA) was used as a standard, and data were expressed as nmol/ml. Total antioxidant capacity (TAC) in seminal plasma and/or serum samples were measured according to the reduction of Fe³⁺ to Fe²⁺. In this experiment, the complex between Fe²⁺ and TPTZ, as an indicator, gives a blue color with an absorbance maximum at 593 nm.^[16] The level of nitrite, an indicator of the production of NO, was determined using Griess reagent (1% sulfanilamide, 0.1% NED, and 2.5% phosphoric acid). Equal volumes of Griess reagent and seminal plasma and/or serum samples were mixed and incubated for 10 min at room temperature in the dark. The absorbance detected at 520 nm using a microplate reader.^[11] Finally, the level of nitrite was assayed from sodium nitrite standard curve and expressed as µg/ml.

Statistical analysis

The data were expressed as means ± the standard error of the mean and analyzed using Graph Pad Prism software, version 6.0. The student's *t*-test was applied to

detect differences between the means of two normally distributed populations. The degree of association between variables was evaluated based on Pearson's or Spearman's correlation coefficient. A value of *P* < 0.05 was considered statistically significant.

Results

Serum analysis

As shown in Table 1, a significant decrease was observed in BChE activity in farmers compared to urban population (*P* < 0.001). In addition, a significant decrease was found in LH serum level (*P* < 0.001), and a remarkable increase was detected in testosterone serum level of farmers (*P* = 0.0014) in comparison with urban region population. No changes were observed in FSH levels between the two groups (*P* = 0.078).

Semen analysis

As shown in Table 2, the number of sperms (*P* = 0.04) as well as their motility (*P* < 0.001) and progressive status (*P* < 0.001) in rural farmers were significantly lower than urban region population. No changes were observed in other indexes such as semen volume (*P* = 0.29), pH (*P* = 0.22), and sperms morphology (*P* = 0.36).

Serum oxidant/antioxidant status

As shown in Figure 1a, although serum NO level increased in rural farmers, these changes were not significant in comparison with urban populations (*P* = 0.27). However, a significant increase was observed in serum LPO level [Figure 1b], and a remarkable decrease was found in serum TAC level of rural farmers [Figure 2] compared to urban people (*P* = 0.0004 and *P* = 0.014, respectively).

Table 1: Reproductive hormones levels and butyrylcholinesterase activity in serum samples of urban and rural men population

Serum biomarkers	Urban (n=30)		Rural (n=30)		P
	Mean±SEM	Minimum-maximum	Mean±SEM	Minimum-maximum	
BChE activity	5862±220.5	930-7559	4406±238	1010-6313	<0.001
LH	5.23±0.36	1.67-8.86	2.98±0.17	1.30-5.70	<0.001
FSH	4.31±0.34	1.4-7.93	3.51±0.28	1.8-7.60	0.078
Total testosterone	5.62±0.40	2.47-10.81	7.62±0.43	4.17-11.65	0.0014

The data were expressed as mean±SEM. The Student's *t*-test was applied to detect differences between the means of two normally distributed populations. *P*<0.05 was considered statistically significant. SEM=Standard error of the mean, BChE=Butyrylcholinesterase, LH=Luteinizing hormone, FSH=Follicle-stimulating hormone

Table 2: Semen analysis of urban and rural men population

Sperm parameters	Urban (n=30)		Rural (n=30)		P
	Mean±SEM	Minimum-maximum	Mean±SEM	Minimum-maximum	
Semen volume (ml)	4.4±0.25	3.1-7.8	4.1±0.21	1.5-5.6	0.29
Semen pH	7.5±0.09	6.6-8.4	7.6±0.08	7-8	0.22
Sperm count (×10 ⁶ /ml)	61.5±6.66	19.2-150.6	43.8±5.56	1.1-137.2	0.04
Motile sperms (%)	73.4±2.8	15.3-81.6	49.8±3.7	10.9-86.2	<0.001
Progressive sperms (%)	64.1±2.5	5.1-84.3	42.5±3.4	5.8-70.2	<0.001
Normal morphology (%)	68.3±2.8	35.2-95.2	71.3±1.5	50.3-91.3	0.36

The data were expressed as mean±SEM. The Student's *t*-test was applied to detect differences between the means of two normally distributed populations. *P*<0.05 was considered statistically significant. SEM=Standard error of the mean

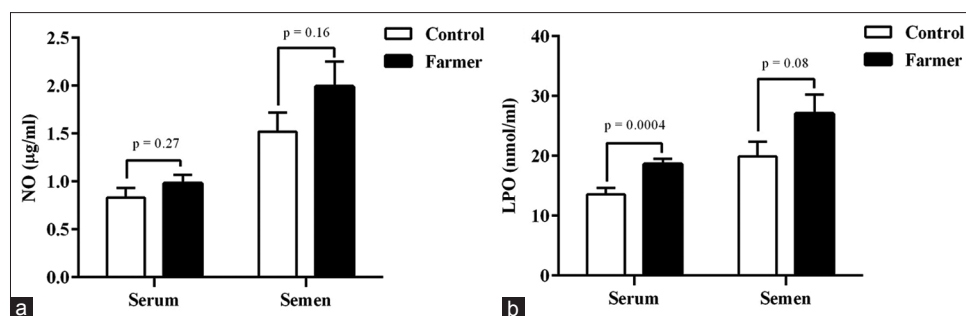


Figure 1: (a) Nitric oxide and (b) lipid peroxidation levels in semen and serum samples of urban and rural men population. Values are expressed as mean \pm standard error of the mean, $n = 30$ for each group. Statistical analysis used Student's t -test. $P < 0.05$ was considered statistically significant

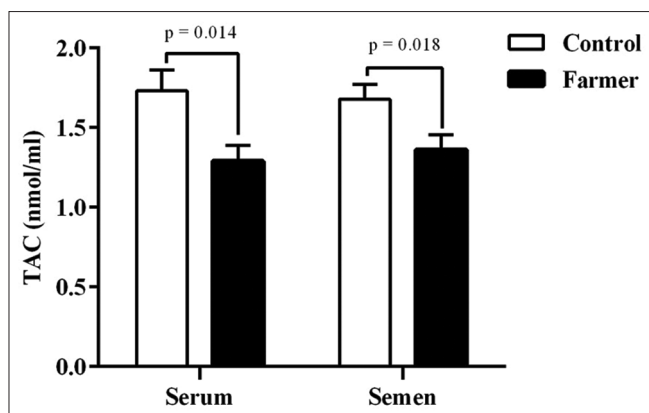


Figure 2: Total antioxidant capacity in semen and serum samples of urban and rural men population. Values are expressed as mean \pm standard error of the mean, $n = 30$ for each group. Statistical analysis used Student's t -test. $P < 0.05$ was considered statistically significant

Semen oxidant/antioxidant status

As shown in Figure 1, the NO and LPO levels increased in rural men semen, but these changes were not significant in comparison with urban region people ($P = 0.16$ and $P = 0.08$, respectively). However, a remarkable decrease was observed in semen TAC level of rural farmers [Figure 2] in comparison with urban region population ($P = 0.018$).

Correlation values – multiple linear regression analysis

We examined the correlation between semen quality parameters and its oxidant/antioxidant status in groups. A positively significant correlation was found between sperm motility and semen antioxidant capacity ($r = 0.45$; $P < 0.05$). However, we did not observe a significant correlation between LPO and NO with semen quality parameters.

Discussion

In the current study, the serum BChE activity, as a biological indicator of OPCs exposure,^[17] was evaluated in both the case and control groups. The mean of BChE enzyme activity was about 25% lower in the rural farmers

than the population in an urban region; this confirms the hypothesis that rural farmers have a greater exposure to OPCs than the control group. In agreement with our data, a decrease in the activity of BChE enzyme has been reported in Pakistan, South Indian farmers,^[18,19] and workers engaged in spraying insecticides.^[20] Nowadays, semen analysis is used to confirm healthy fertility in men. Our findings denote a considerable decrease in sperm levels in a rural population, which was also reported by Hossain *et al.* in Malaysia.^[21] In addition, the animal studies have confirmed a reduction in spermatogenesis in relation to OPCs toxicity.^[22] It has been suggested that OPCs, such as chlorpyrifos, methyl parathion, and parathion, can affect sperm levels by injuring the seminiferous epithelium through germ cell proliferation.^[22,23] Furthermore, OPCs could cross the epididymal epithelium due to its lipophilic features and reach the stored spermatozoa would explain its destructive effects on sperm structure and function.^[24]

However, infertility seems to be caused by a lower sperm count that can be seen by exposure to OPCs; that is, as a whole, a sperm count of $<20 \times 10^6$ /ml results in infertility, while the average sperm count in farmers has been reported to be twice that of the lowest amount required for fertility.^[25]

Sperm motility depends on the integrity of the sperm's tail and midpiece to generate energy to move. In addition, adenosine triphosphate (ATP) is the major source of energy for viability of spermatozoa.^[26] Therefore, any factor interfering with the ability to assemble tail structure proteins and/or modify ATP synthesis can lead to decreased sperm motility. In the current study, the sperm motility level was lower in rural farmers than men in urban areas (the control group). In previous studies, metabolic modifications have been reported to occur following exposure of various tissues to OPCs, such as changes in the glycolytic pathway and alterations in mitochondrial respiration.^[17,27] It seems that mitochondrial dysfunction can decrease ATP synthesis and cause a significant decrease in sperm motility.

Oxidative stress can have a negative impact on sperm production and gonadal function. In the current study, the occurrence of oxidative stress was confirmed in rural farmers by decreased TAC level in serum and semen samples as well as an increased in serum LPO levels. However, the nitrite levels, as indexes of the nitrosative stress pathway, did not change in the semen samples in the two groups. The ROS are generated by two main pathways. In the first pathway, the ROS may be generated as the result of the biotransformation of OPCs by cytochrome P450 enzymes. These enzymes create ROS by addition of one atom of molecular oxygen into a substrate (such as OPCs) by an electron transport pathway.^[28] In the second pathway, following OPCs exposure, high ATP consumption coupled with inhibition of oxidative phosphorylation, leads to decreased ability of cells in maintenance of its energy levels. Therefore, the excessive amounts of ROS may be produced in various cells.^[29] Among these, sperm cells are very sensitive to oxidative damage due to their structural properties. For example, docosahexaenoic acid is one of the major fatty acids in the membrane structure of spermatozoa cells that predisposes the membrane of these cells to oxidative stress damage.^[30] The reaction of free radicals with membrane lipids leads to the formation of bioactive aldehydes, such as MDA.^[17] These products may retard the maturation, decrease in number, and motility of sperm cells.

In the current study, a positive correlation between decreased antioxidant capacity and sperm motility was found in rural farmers. In agreement with our results, Khosrowbeygi *et al.*^[31] reported on this correlation between semen quality and antioxidant capacity. A change in enzymatic antioxidant activity, such as catalase and superoxide dismutase, can be the underlying etiology of this correlation.

Spermatogenesis is basically controlled by sex hormones. LH secreted by hypophysis induces testosterone production in sertoli cells and impacts their development.^[32] Several studies have been reported that the pesticides might effect on sex hormone production. For instance, Melgarejo *et al.*^[6] reported that there is a significant positive association between diethyldithiophosphate concentrations and LH as well as FSH levels. Miranda-Contreras *et al.*^[7] conducted a case-control study in male Venezuelan and reported a tendency for increased LH and FSH levels in farm workers when exposed to pesticides. In the other case-control study, Waheed *et al.*^[19] found that exposure to pesticides is related to high testosterone level in occupational and residential users of pesticides. In the current study, despite an increase in testosterone levels, the LH level was significantly decreased in the study group (the farmers). It seems that OPCs can affect these

hormones by two completely different mechanisms. The studies have shown that OPCs such as malathion and chlorpyrifos, impact testosterone metabolism by inhibiting CYP3A4, resulting in an increase in serum testosterone and its perpetuity.^[33,34] However, low serum LH levels can be explained by OPCs' effects on hypothalamic-pituitary endocrine functions. It is assumed that OPCs can alter hypothalamic-pituitary endocrine functions by decreasing the AChE activity in the brain. As a result of AChE inhibition, OPCs may significantly increase acetylcholine level, and monoamine levels such as epinephrine, norepinephrine, 5-hydroxytryptamine, and dopamine concentrations, ultimately affecting secretion of pituitary hormones.^[35]

Conclusion

Our findings showed that oxidative stress, increased testosterone levels and decreased LH can all be essential underlying factors in the lower quality of semen in the farmers. However, this decreased quality of semen and decreased sperm count cannot definitively explain the infertility in this population; thus, further studies are needed to investigate the reasons for infertility in the farmers of the rural region, Hamadan, Iran.

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Conflicts of interest

There are no conflicts of interest.

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