Sperm nuclear deoxyribonucleic acid denaturation in diazinon/diazoxon sprayer men

Sherif MH El-Kannishy(PhD)[1], Rizk M El-Baz(PhD)[2], Soma Sh Abd El Gawad(MD)[3], Hamdy F Marzook(MD)[3], Samia A Hassan(MD)[4],and Abdelhamid A Metwali (MD)[5]

[1] Analytical Toxicology - Emergency Hospital, [2] Genetic Unit – Pediatric Hospital, [3] Clinical Pathology Dept., [4] Forensic Medicine & Clinical Toxicology Dept., and [5] Internal Medicine Dept., Faculty of Medicine, Mansoura University, Egypt. somaabdelgawad@yahoo.com

Abstract: Objectives: Excessive exposure of agrochemical male workers to organophosphate (OP) pesticides may induce morphofunctional changes in their sperms**.** The aim of this study was to explore sperm nuclear deoxyribonucleic acid (DNA) reaction to in vitro incubation with or in vivo chronic exposure to diazinon or diazoxon. **Methods:** Fixed volumes of every semen sample of ten healthy volunteers were incubated at 37°C for one hour with rising serial volumes of 60% of either diazinon (DZ) or diazoxon (DZO). Induced sperm morphological alterations were determined by microscopic examination of direct fresh, Papaniclaue stained and eosin-Y exclusion smears while, sperm nuclear cytotoxicity was assessed by DNA fluorometric examination. On the other hand, sperms of 20 chronic agriculture DZ/DZO spraying workers were directly examined for their quality and DNA denaturation state. **Results:** Normal human spermatozoa showed unfavorable increasing alterations in their quality and DNA integrity after their incubation with serially rising volumes of either diazinon or its oxon. Induction of sperm nuclear DNA denaturation by DZO was more severe both qualitatively and quantitatively than after DZ treated testing. Similar alterations but to lesser extent were found in sperms' DNA of chronic DZ/DZO spraying workers without exogenous OP treatment. **Conclusion:** In vivo DZ/DZO chronic exposure induced unfavorable effects in seminal quality and sperm DNA integrity but were lesser in strength than in vitro testing.
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Introduction:

Organophosphates (OPs) are among the most world-wide used agrochemical pesticides. Among them, diazinon [O, O-diethyl O-2-isopropyl-6-methyl-pyrimidine 4-yl-phosphoro-thioate] is a synthetic highly toxic OP compound with broad-spectrum pesticide activities. Diazinon (DZ) spraying workers intoxication occurs through its oxon metabolite diazoxon (DZO) which inhibits acetyl cholinesterase (AChE) bioactivity resulting in accumulation of acetylcholine. This latter compound induces pathophysiological manifestations mainly at nerve synapses [1]. In addition, non-neurotoxic OP harmful bioactivities are noted in several tissues among which are the testes [2-5]. Since DZO has alkylating and clastogenic activities, it can induce genetic, mutagenic and carcinogenic pathologies [6]. However, the potential toxicity of many OPs on somatic and germinal cells are not yet completely settled [7].

Normal sperm nuclear DNA integrity is naturally preserved by its highly compacted structure, but it may be denatured after extensive or prolonged exposure to organophosphates [8]. Chromatin condensation in the sperm nucleus is a relevant factor for reproduction and a sensitive indicator of male fertility. Altered sperm chromatin structure has been reported in some OPs exposed agriculture workers[2,3,7-9] and experimental animals[2,4,10]. However, Salazar-Arredondo and colleagues [11] reported that, incubation of human spermatozoa with diazinon, chlorpyrifos and methyl parathion or their oxons (up to 750 micromol concentration) did not induce sperm cytotoxity or DNA fragmentation. Moreover, the noted untoward spermatozoal cellular membranes reactions in such toxicity are related to their high content of polyunsaturated fatty acids and lack of intracellular antioxidants and nuclear DNA-repair systems [12].

Organophosphate exposure can induce sperm nuclear chromatin de-condensation and cell membrane lipid peroxidation along sperm passage through the male genital tract [12]. Clinically, exposure to organophosphate insecticides may compromise male fertility and induce anomalies or diseases in the off springs. When the extent of DNA damage is small, the affected spermatozoa can undergo self-repair. Also the oocyte is capable of repairing damaged DNA in these spermatozoa. Alternatively, if the damage is extensive, apoptosis, fragmentation and losses of the embryos can occur [13,14].

Aim: To explore sperm nuclear deoxyribonucleic acid (DNA) reaction to in vitro

incubation with or in vivo chronic exposure of their donors to diazinon or diazoxon.

Subjects and methods:

The subjects of the present study comprised:

(1) Twenty spraying workers in northern Nile-Delta States of Egypt, with intermittent (three times/week) DZ-DZO spraying duties for > 5.0 years.

(2) Ten healthy fertile individuals with no history of OP direct exposure. Their semen samples were used as a normal unexposed reference material and for in vitro determination of sperm vitality and DNA fragmentation index (DFI%) after incubation with rising concentrations of DZ/DZO. Normal semen should have normal quantitative and qualitative parameters [15] and their donors fathering children.

After obtaining an informed signed consent from each participant in both groups, a semen sample per individual was collected by masturbation into a sterile container with 3-4 days abstinence before sampling. Thereafter, all semen samples were subjected to the following:

(a) Morphofunctional examination:

Each semen sample was examined within an hour of collection by using a Computer Assisted Sperm Analyzer [Weili Color Semen analysis System – ALTY – 9000, China].

(b) In vitro effect of rising DZ/DZO concentrations on sperm vitality %:

A designed volume of each ejaculate obtained from every healthy fertile individual was diluted with saline to get 50 000 sperm/ml. Then 20 µl of each diluent, which contained 1000 sperms were distributed into different tubes in two separate rows, each comprised 10 tubes. One row for DZ (60% w/v active DZ ingredient) while, the second row for DZO after its dilution by saline to obtain 60% solution. To each of the first eight tubes of either row, one of the OP compound was added in serial rising volumes of 100, 200, 300, 400, 500, 600, 700 and 800 ul. Then to all these tubes in the two rows, 0.15% DMSO (Sigma) solution was added to complete their final volumes to 900 ul. Also to each tube within the two rows, 0.6 ml bovine albumin suspensions (3.5 mg/ml) were added. All these tubes were incubated for 1.0 hr in a water bath at 37°C. Morphofunctional characteristics of the spermatozoa after their incubations were evaluated according to the WHO guidelines [15]. Also, sperm vitality% using 0.5% eosin Y was assessed and expressed as percentage by counting dye excluded sperms among 100 spermatozoa.

A negative control (tube 9) for each semen sample containing 1000 spermatozoa diluted to 900 µl with 0.15% DMSO solution and completed to 1.5 ml by 3.5 mg/ml bovine albumin was prepared without DZ or DZO addition. Then these seminal preparations were managed as above. Also, a positive control (tube 10) containing similar constituents as in the test tubes but instead of the organophosphate, H_2O_2 (100 micromol) was added and similarly managed.

(c) In vivo and in vitro assessment of DZ-DZO toxic effects on spermatozoal nuclear DNA by sperm chromatin structure analysis (SCSA) [16-19]:

Within 4 hours from ejaculation, aliquots of the different semen samples, each contained 2.0 million spermatozoa were kept frozen at -70ºC. On the day of DNA analysis, the stored samples were thawed in a 37ºC water bath. It is established that analysis of frozen/thawed semen samples gave equivalent results to those from fresh samples.

Sperms were analyzed using FACSort flow cytometer (Beckton Dickinson, San Jose, Canada), equipped with an argon ion lazer (488nm). The flow cytometer was calibrated for each measurement session (10 semen samples) using reference frozen semen samples for flow cytometer set up and as an internal quality control, with a variation coefficient <5% and a low level sperm DNA damage (the red and green photo-multiplier yielded the same red and green fluorescence levels i.e 130/1000 and 500/1000 channels $\pm$ 5.0).

Semen samples were diluted with TNE buffer (0.01 mol Tris HCl, 0.15 mol NaCl and 1 mmol EDTA) to obtain 1000000 sperms/ ml. Then 200 µl aliquots of each diluent (containing 200 000 sperms) were mixed with 400 µl of a low pH (pH 1.2) detergent solution containing 0.1% Triton X 100, 0.15 mol NaCl, and 0.08 mol HCl and left for 30 seconds. This was followed by staining with 1.2 ml of 6 µg/ml chromatographically purified acridine orange (AO) [Polysciences, Washington, PA] in a phosphate-citrate buffer (pH 6.0). Three minutes after staining, specimens were analyzed by the flow cytometry. Measurements were performed in duplicates; each contained 5000 sperms (45 µl of the final specimen) at a flow rate of less than 200 sperms/second. The fluorochrome AO that, intercalated into double-stranded DNA (native) fluoresced green (515-530nm), while AO that intercalated into single-stranded DNA (denatured) emitted red fluorescence (<630nm).

The extent of DNA denaturation was quantified by the DNA fragmentation index (DFI) which represented the shift from green to red fluorescence i.e. the ratio of red to total (red + green) fluorescing sperms. The calculated DNA fragmentation index% (DFI%) is a reliable measure of fertility[19].

Statistical analyses:

The Statistical Package for Social Scientists (SPSS) for windows program Version 11 (SPSS Inc., Chicago, USA) was used for statistical calculations.

In vitro DZ or DZO treated semen samples of healthy volunteers were compared by analysis of variance with t-test. Similarly, native semen of DZ-DZO spraying workers was statistically analyzed. Sperm DNA fragmentation index% (DFI%) of the in vitro DZ/DZO treated and in vivo DZ-DZO chronically exposed sprayers as well as in the native healthy untreated sperms were assessed. P<0.05 values were considered statistically significant.

Results:

Table (1) shows seminogram of OP-unexposed (normal volunteers) and DZ/DZO exposed sprayers. Only 20% of patients had normal semen characteristics while, the remaining 80% had one or more seminal abnormality of the standard semen parameters.

Table (2) shows the percentage of forward motility% (viability or vitality %) of sperms. It was significantly decreased after sperms incubation with rising volumes of DZ/DZO. Also, SCSA measurements are shown in this table. Sperm DNA structure was altered in samples obtained from the OP agriculture spraying workers.

Among the DZ/DZO sprayers, 20% of the examined semen samples present infertility potential (≥30% DFI percent), whereas the remaining cases were potentially subfertile (<30% DFI percent). However, two wives suffered from miscarriage when pregnancy occurred.

In vitro testing (using sperms of OP-unexposed healthy volunteers) declared that the percentage of sperms showing DNA denaturation (fragmentation index %) was significantly higher with DZ treated (14.0±4.0% - 36.6±11.8%) than native untreated sperms (10.8±3.7%). The degree of DNA damage increased according to the DZ concentration. DFI% (s) range after sperm incubation with DZO (14.7±4.1 – 39.6±10.5%) was not significantly different from those incubated with DZ similar concentrations (Table 2 & Figure 1). Clinically the severity of sperm toxicity was higher among chronic sprayers with DZO than those using DZ.

With exception of seminal volumes and counts, significant positive correlations were observed between percent of other change of conventional semen quality parameters and SCSA determined DFI%.

Table (1): Semen analytical parameters (X±SD) of the two investigated groups (OP non exposed volunteers as control versus DZ/DZO spraying workers) within one hour after ejaculation.

Variables	**Non exposed volunteers** (n:10)	**Spraying workers** (n:20)
Volume (ml)	3.3±0.9	2.9±0.6
Viscosity	Normal	Less than normal
Count (million/ml)	56.3±14.8	40.5±12.1*
Progressive motility (viability) %	77.8±10.6	59.7±11.6*
Abnormal Morphology %	19.5±4.1	28.7±5.6*
Round head	8.9±1.5	14.1±2.2*

* Significant difference from normal values. Data: mean ± SD

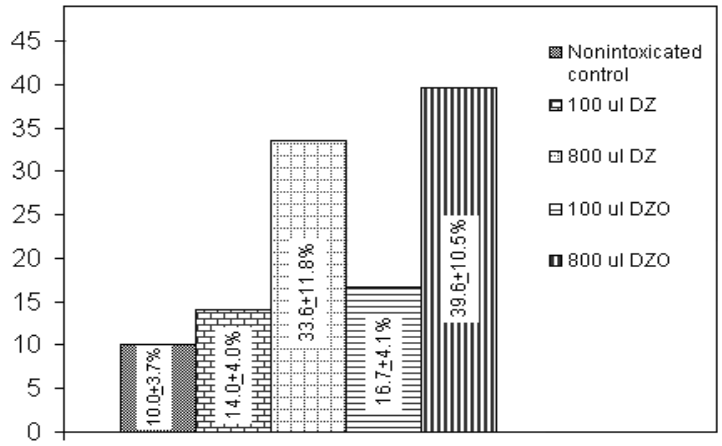

Figure (1): Minimal and maximal DFI% by DZ/DZO in vitro testing.

Table (2): Spermatozoa vitality % and DFI% values non exposed (native) control, DZ/DZO spraying workers and after incubation with rising volumes of DZ or DZO for one hour.

Semen donors		Sperm vitality %	DNA fragmentation index % (DFI%)
(1) Non-intoxicated healthy control (10 individuals)		77.8±10.6	10.8±3.7
(2) Chronically exposed spraying workers (20 workers; of them 4 cases had DFI% ≥ 30%)		59.7±11.6	18.0±5.6*
(3) In vitro incubation of normal semen with 60% DZ/DZO:			
100µL	DZ	61.0±11.9	14.0±4.0
	DZO	58.0±13.5*	16.7±4.1
300µL	DZ	56.9±15.1*	17.1±7.5
	DZO	46.5±11.0*	22.1±6.8**
500µL	DZ	50.5±14.7*	22.0±7.5**
	DZO	40.8±11.3*	29.3±8.4**
700µL	DZ	44.6±13.0*	28.9±9.1**
	DZO	33.1±10.6*	34.4±10.6**
800µL	DZ	35.4±10.8*	33.6±11.8**
	DZO	30.9±9.9*	39.6±10.5**

* Significant difference compared to the control ($p < 0.05$).

* * Significant difference between DZ and DZO and control (p : <0.001).

Discussion:

Organophosphates can affect spermatogenesis through altering sperm chromatin structure and DNA integrity. Increased sperm DNA denaturation in situ reflects DNA strand breaks and nuclear maturity delay [4,6,8,10]. In male reproductive system, sperm DNA susceptibility to OP denaturation and potential mutagenicity are dependent on the spermatogenic cell stage at time of exposure [5]. Toxicity of different OPs on somatic cells showed contradictory results and OP effects on human male germinal cells need extended investigations [8,12].

The present study showed that, only 20% of the evaluated patients had normal semen characteristics while the remaining had one or more abnormality of the standard semen parameters (Table 1). In vitro DZ-induced cytotoxic effects on mature sperm cells were quantitively and qualitatively less than those noted after DZO similar concentrations (Tables 1&2). This difference reflected the rate of conversion of the biologically inactive DZ to its active DZO intermediate. In this respect, it has been reported that agriculture OP spraying men had high concentrations of OP pesticides in their blood and body organs including the reproductive system [3,5]. Moreover, this later system can generate highly toxic reactive oxygen species (ROS) in situ [20,21] which could intensify testicular dysfunction. In the epididymis, spermatozoa of OP exposed individuals are subjected to induced oxidative stress due to increased ROS and lipid peroxidation with reduced antioxidant enzymes and glutathione [4,6,20-23].

The present study showed that induced sperm DFI% by in vitro DZ or DZO treatment was significantly higher than that with OP unexposed control testing. These in vitro DZ/DZO effects on DFI% were dose dependent (Tables 2 and Figure 1). However, DFI% after in vitro sperm incubation with rising DZO content were higher but not significant than the corresponding DZ activity. It has been reported that injection of mice with a single dose of DZ (8.12 mg/kg) increased DFI and DFI% values due to decreased sperm chromatin condensation and increased DNA denaturation during spermatid differentiation[4]. On the other side, in vitro incubation of sperms with DZ/DZO for one hour was more deleterious than the corresponding intermittent chronic OP exposure of spraying workers (Table 2). This may be due to natural human biological responses such as degradation and clearance of OPs. However, Salazar – Arredondo et al.,[11] found that incubation of normal spermatozoa with DZ or DZO at different concentrations could not induce significant sperm cytotoxicity as evaluated by eosin-Y exclusion.

Chronic exposure to OP oxons can alter sperm chromatin quality and even induce its damage in agriculture OP sprayers [3,5,7-9] and experimental animals[2,4,10]. These spermatozoal reactions were related to their high content of polyunsaturated fatty acids particularly arachidonic acid in their plasma

membranes and relative lack of intracellular antioxidants and nuclear DNA-repair systems [12].

Incubation of semen samples with ≥700ul DZ or 500ul DZO for one hour induced ≥30% of DFI% (Table 2) as shown by high proportion of red fluorescence (denatured single strand DNA) with concomitant low green fluorescence (native double strand DNA). So, OP oxons alter sperm DNA integrity at higher degree and faster rate than their parent compounds. In this respect, OP toxic activity varies from one OP to another. However, the toxic potential of DZO was considered of moderate severity level compared with other OP insecticides [4,10].

Sperm axonemal protein phosphorylation induces reduction of membrane fluidity that is necessary for sperm-oocyte fusion. Oxidative stress can lead to sperm damage, deformity and eventually male infertility. The tight packaging of sperm nuclear DNA and the physiological antioxidants content in seminal plasma protect spermatozoal DNA from oxidative stress induced damage. The high frequencies of single- and double-strand DNA breaks, gene mutation and polymorphism, result in decreased semen quality and increased DNA denaturation and DNA base – peroxidation [13,14].

Infertile men possess substantially more sperm DNA damage than do fertile men. Such damage may impact negative reproductive outcomes [24]. DNA damage involves an error in chromatin remodeling during spermatogenesis, leading to generation of spermatozoa with subnormal protaminated nuclear DNA. Close relationships exist between the efficiency of chromatin remodeling and oxidative DNA damage and fragmentation in human spermatozoa [25-27].

Conclusion:

In the present study, significant negative correlations were observed among the clinically important absolute semen quality values (sperm forward motility % and vitality %) and SCSA DFI %. Alternatively, significant positive correlation between DFI% and absolute abnormal morphology% was found.

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