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Full Length Research Paper

Frequently-used agrochemicals lead to functional and morphological spermatozoa alterations in rats

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It is known that agrochemicals alter male reproductive functions. Previous studies from our lab have demonstrated the relationship between male fertility dysfunction and oxidative-nitrative stress. In this work, morphological and functional spermatozoa parameters were studied in a rat model sub-chronically (5 weeks) intoxicated with low doses (i.p. 1/250 LD_{50}) of a mixture of dimethoate (D), glyphosate (G) and zineb (Z). The cytological assays showed alterations in spermatozoa morphology and in plasma membrane integrity. Modifications in the fatty acid composition were also shown. RIA analyses demonstrated androgenic hormone imbalance in plasma and testes. The acrosome reaction was also altered. Free thiols (positively correlated with DNA denaturation) and fructose levels were elevated in seminal vesicles from treated rats. Taking into account the low doses of pesticides that provoke these alterations, it was assumed that the environmental pollution may play a key role as a causative factor for fertility abnormalities.

Key words: Dimethoate, zineb, glyphosate, rat, spermatozoa, fertility/sperm abnormalities.

INTRODUCTION

Health disorders associated with environmental pollution are a cause of international concern. Among them, the incidence of male fertility disturbance has been increasing since the 1980's probably due to multi-factorial events involving both environmental and genetic factors (Giwercman et al., 1993; Petrelli and Mantovani, 2002; Pflieger-Bruss et al., 2004). Several studies concerning the effect of agrochemicals on the reproductive system suggest various possible mechanisms of toxicity. The most documented ones are the hormonal disruption (Sarkar et al., 2000; Bhatnagar, 2001; Basrur, 2006; Joshi et al., 2007) and the alteration of the antioxidant defense system (Sikka, 2001; Sheweita et al., 2005; Aitken and Baker, 2006; Kesavachandran et al., 2009).

So far, pesticides are widely used for agricultural purposes. Thus, residues of many of them (and possibly their metabolites) could remain as pollutants in water, air and food. In this work, we studied three of the most commonly used pesticides worldwide: zineb (Z), glyphosate (G) and dimethoate (D) in combination. Dimethoate is an organophosphorus insecticide of systemic action extensively used in pest treatment in onions, tomatoes, and citric fruits among others (Sharma et al., 2005b). Glyphosate is a systemic herbicide used to

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Abbreviations: c, Control group; **D,** dimethoate; **FSH,** folicle-stimulating hormone; **G,** glyphosate; **LH,** luteinizing hormone; **OPs,** organophosphorus pesticides; **OS,** oxidative stress; **PEG-400,** polyethyleneglycol-400; **RNS,** reactive nitrogen species; **ROS,** reactive oxygenated species; **ZGD,** treated group; **Te,** testosterone; **Z,** zineb.

control undergrowth before seeding in corn, soybean, vine, etc. (Daruich et al., 2001) and zineb is a contact fungicide used to control pests in carrots, onions, citric fruits and potatoes (Heikkila et al., 1976).

It was previously reported that dimethoate impairs spermatozoa motility, decreases serum testosterone levels and testicular weight, and increases the percentages of dead and abnormal spermatozoa in rats, rabbits (Salem et al., 1988; Walsh et al., 2000b; Afifi et al., 1991) and mice (Farag et al., 2007). Moreover, a previous work from our lab demonstrates that, dimethoate displays a complex mechanism of action involving disturbances in the hormone production (at both systemic and Leydig cell levels). We found alterations in the antioxidant defense system, decreased phospholipids araquidonate content, inhibition of StAR protein expression with simultaneous stimulation of COX-2 (overproduction of PGF2α) and also the inhibition of steroidogenic enzymes 17βHSD and 3βHSD (Astiz et al., 2009b).

Recent studies in rats suggest that, the exposure to glyphosate during the pre- and post-natal periods induces adverse effects on male reproductive performance (Dallegrave et al., 2007). The exhaustive bibliographic revision made by Basrur (2006) showed that, glyphosate can act as a sexual differentiation disruptor and as an estrogen-like compound in domestic animals and humans. Moreover, epidemiological evidence indicates that women which couples were in contact with glyphosate had difficulty to conceive and also showed a higher rate of miscarriage (Arbuckle et al., 2001; Caglar and Kolankaya, 2008). In addition, zineb produces a decrease in mouse fertility performance due to alterations in male and female pronuclei formation (Rossi et al., 2006).

Traditionally, male infertility diagnosis depends on microscopic and biochemical assays to assess spermatozoa concentration, morphology, and motility. Over the past decades, several *in vitro* tests have been developed to evaluate aspects involved in sperm functional competence which included movement ability, cervical mucus penetration, capacitation, acrosome reaction, sperm-oocyte fusion, redox status, and integrity of nuclear and mitochondrial DNA. These methodologies should be useful to predict either *in vitro* or *in vivo* sperm fertilizing ability with relatively high accuracy (Aitken, 2006; Lewis, 2007). However, most (if not all) investigations mentioned earlier concerning reproductive toxicity, were performed in single intoxication animal models using high doses (near LD_{50}) of pesticides rarely observed in daily exposure. For this reason, the present work was undertaken to evaluate the chronic effect of low doses (1/250 LD_{50}) of the aforementioned agrochemicals administered, as a mixture. The doses selected for our experiments were chosen according to previous reports on their toxic effects (John et al., 2001; Sharma et al., 2005a; Beuret et al., 2005; Nielsen et al., 2006; Patel et al., 2006).

Our aim was to explore the impact of such association in testicular performance by a more realistic experimental model, because many pollutants reach our tissues as mixtures present in environment to which animals and humans are inevitably exposed during prolonged periods (Cory-Slechta, 2005). Our specific targets were (i) to evaluate alterations in the morphological and functional sperm characteristics, and (ii) to study biochemical parameters related with fertility performance such as capacitation and acrosome reaction, hormonal levels, and thiol and fructose contents. The results may contribute to the risk assessment of involuntarily and chronically pesticide-exposed populations.

MATERIALS AND METHODS

Chemicals

Most chemicals used were of reagent grade and obtained from Sigma Chem. Co. (CA, USA, or Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). Organic solvents were from Carlo Erba (Milano, Italy). Other chemicals were purchased from local commercial sources and they were of analytical grade. The pesticides, dimethoate (O,O-dimethyl-S-methyl-carbamoyl-methyl phosphorodithioate), zineb (zinc ethylene-bis-dithiocarbamate) and glyphosate (N-phosphonomethyl-glycine) were obtained as a gift from Instituto Nacional de Tecnología Agropecuaria (INTA, National Institute for Agrochemical Technology, Castelar, Argentina).

Animal care and treatment

Male Wistar rats weighing 190 ± 20 g were breaded by the Laboratory Animals service from the Veterinarian School at La Plata National University, they had a certified pathogen-free status. Before starting with the experiment, they were allowed to acclimatize for a week. The rats were maintained under controlled temperature (25 ± 3°C), and with a normal photoperiod of 12 h darkness and 12 h light. They were fed with standard Purina chow from Ganave S.A. (Santa Fe, Argentina) and given water *ad libitum* in agreement with the American Institute of Nutrition (Reeves et al., 1993). Clinical examinations and body weight evaluations were performed every week during the experiment. The animals were in very good conditions along the experimental period. No sign of toxic effect was observed. None of the treatments influenced water consumption (approx. 15 ml/day), final body weights, body weight gain rate, food efficiency ratio, and testicular weight (Table 1). Pesticide exposure did not affect the animal behavior. Also, no visible signs of toxicity and/or cholinergic effects were observed during the entire experimental period. There was no mortality.

The animals were randomly divided into two groups of five animals each, assigned as control rats (C) injected intraperitoneally (i.p.) with polyethyleneglycol-400 (PEG-400), and treated (ZGD) rats injected i.p. with a combination of 15 mg dimethoate (D)/kg of body weight (bw), 15 mg zineb (Z) /kg bw and 10 mg glyphosate (G)/ kg bw dissolved in PEG-400. All animals were injected three times a week for five weeks. Dosing schedule was selected in view of previous experimental protocols used by us and other researchers of the area (Bagchi et al., 1995; John et al., 2001;

Table 1. Main feeding parameters associated to experimental treatments.

Parameters	Treatments	
	C	ZGD
Initial body weight (g)	181.0 ± 3.7	174.0 ± 7.3
Final body weight (g)	307.5 ± 13.7	288.8 ± 20.9
Body weight gain (g)	126.5 ± 3.4	114.8 ± 5.5
Rate of body weight gain (g/day)	3.6 ± 0.1	3.3 ± 0.2
Food efficiency ratio[a]	9.5 ± 0.2	8.7 ± 0.3
Absolute testicular weight (g)	2.8 ± 0.1	2.9 ± 0.1
Relative testicular weight (mg/g)[b]	9.1 ± 0.3	10.0 ± 0.4

C, Control rats; ZGD, treated rats. Values represent the mean ± SD (n = 8). [a] Food efficiency ratio = [body weight gain (g) / food intake (g)].10^2. [b] Relative testicular weight = testis weight (mg)/ body mass (g).

Nielsen et al., 2006; Sivapiriya et al., 2006; Astiz et al., 2009a, b, c and d). We think that such low doses would be reached in certain circumstances, especially in rural zones exposed to frequently agrochemical spraying from airplanes, or even by ingestion of fruits and vegetables not previously analyzed. Most of the previous reports in the field of pesticides and oxidative stress were performed in single intoxication animal models. For this reason, the present investigation was undertaken to evaluate the effect of a sub-chronic exposure to low doses of agrochemicals administered in combination. This may contribute to the understanding of the effect of a simultaneous exposition, and it may have interest for the evaluation of the damage degree to which humans are potentially exposed due to environmental pollution in a more similar way as it happens in real life exposure. Of course, it is impossible to reproduce exactly what happens to humans; however, with this model we can compare at least the effects of a frequent mixture. We did not pretend to establish a toxicosis with clinical manifestation.

The aim of the study was to explore the effect of sub-clinical and sub-symptomathologycal doses of the agrochemicals tested. So, clinical examinations were performed in order to exclude any sign of toxicosis. A veterinarian observes for the presence of miosis, mouth smacking, salivation, or lacrimation. The rats were also placed in an open field for observation of tremors or gait abnormalities according to Moser et al. (2006). There were no significant differences between controls and treated animals.

Animal maintenance and handling were in accordance with the NIH guide for the care and use of laboratory animals published in 1985. All procedures were approved by the local laboratory animal bioethical committee of Facultad de Ciencias Médicas (UNLP, Argentina).

Sample collection

At the end of the treatment all rats were killed by rapid decapitation. Blood was collected using heparin as anticoagulant (10 UI/ml) and plasmas were immediately prepared by centrifugation (4000 g, 10 min) and stored at -80°C until analyzed. Both testicular epididymis were quickly excised and the caudal zones were transferred into flasks containing 8 ml of Biggers, Witten and Whittingham medium (BWW) (Biggers et al., 1995).

Tissues were minced and incubated (35°C, 20 min) under gentle and constant shaking. Under these conditions epididymal content was spontaneously released into the medium minimizing cell damage (Klinefelter et al., 1991). After spontaneous decanting (10 min) the remaining tissue was removed, while cells were transferred into plastic tubes and centrifuged (700 g; 15 min). Pelleted spermatozoa from both experimental groups were washed twice with BWW and resuspended in 10 ml fresh medium. Both seminal vesicles of each rat were excised, washed and homogenized (glass/teflon homogenizer) with 3 ml ice-cold phosphate buffer 100 mM, pH 7.40 with 6 mM of EDTA. Homogenates were stored at -80°C until analyzed.

Spermatozoa morphology evaluation

Morphology was evaluated microscopically as described by Filler (1993). We studied epididymal spermatozoa shape and morphology by examination of head, mid-piece and tail sections. The staining procedure was similar to the one described by Larson and Miller (1999). In brief, aliquots (1 ml) from each spermatozoa suspension were incubated (25°C, 60 min) with 100 µl of eosin-Y (1% w/v in NaCl 0.9%). Smears were prepared, air dried, covered with Biopack (Synthetic Canadian Balsam 130305 from Biopack S.A., Buenos Aires, Argentina) and a cover slip. Five microscopic fields of each slide (approx. 500 cells each) were analyzed using light microscope (400 X). Spermatozoa were classified as follows: N, normal (without alterations); MZ, with altered head and/or middle section; F, flagellum or tail alterations; or M, mixed (combined) alterations. Results were expressed as percentage of the total spermatozoa count following the normal morphology criteria defined by World Health Organization (1999).

Spermatozoa vitality and plasma membrane integrity

Spermatozoa vitality was estimated using the eosin-nigrosin (EN) supravital stain technique as described by Eliasson and Treichl (1971). 100 µl of each spermatozoa suspension was gently mixed with 100 ml of eosin-nigrosine solution (eosin-Y 5% (w/v) and nigrosine 10% (w/v) in NaCl 0.9%). This dye exclusion technique differentiates vital spermatozoa from dead. Intact spermatozoa (considered alive) were not stained, while spermatozoa with altered cell plasma membrane integrity (considered dead) took up the dye. Smears were prepared on acetone-pretreated slides repeatedly washed with distilled water. They were dried at 37°C and then observed under light microscope (400 X). At least two fields/slide (200 cells/field) of each sample were analyzed and classified as follows: alive (non-stained); dying (partially stained) or dead (completely stained).

Spermatozoa capacitation and acrosome reaction *in vitro*

Spermatozoa capacitation and the acrosome reaction are both essential processes for oocyte fertilization. Acrosome reaction could be induced *in vitro* by adding the Ca-ionosphore A-23187 (Ionomicine) according to the protocol described by Beitbart and Naor (1999). 1 ml of each spermatozoa suspension in BWW containing Bovine Serum Albumin (BSA) 4 g/L and sodium bicarbonate 25 mM was treated with 1 µl of Ionomicine (1 µM final concentration). An equivalent aliquot of each suspension (in BWW free of BSA and sodium bicarbonate) was incubated without Ionomicine as control assay. After a 30 min-incubation (at 35°C),

sub-aliquots (0.5 ml) were taken and mixed with 25 µl of formaldehyde 40%. The remaining suspensions were incubated for further 30 min and then mixed with 25 µl of formaldehyde 40%. All samples were centrifuged (1000 g, 10 min) and fixed cell pellets were resuspended in PBS (0.5 ml). Smears were prepared and stained with Coomasie Brilliant Blue G-250 (0.22% in methanol 50% with 10% of concentrate acetic acid) for 2 min, washed with distilled water, mounted with Biopack® and observed under light microscope (400×). The spermatozoa that underwent acrosome reaction had the head zone unstained, while those without acrosome reaction were completely stained in blue. Results at both both incubation times (30 and 60 min) were expressed as percentage.

Analytical determinations

Fatty acid composition of caudal epididymal spermatozoa

Since spermatozoa lipid composition plays an important role for a successful fertilization, we analyzed the fatty acid (FA) composition of total lipids obtained from caudal epididymal spermatozoa of control and treated rats as described by Aksoy et al. (2006). An aliquot of the spermatozoa suspension (1 ml) was centrifuged (1500 g; 5 min). 5 ml of Folch reagent was added to the pelleted cells (Folch et al., 1957) and shook vigorously for 1 min.

After partitioning, the extracted lipids were saponified and esterified under N_2 atmosphere to obtain the corresponding fatty acyl methyl esters (FAMEs) using boron trifluoride (14%) according to Morrison and Smith (1964) with minor modifications, as described in detail in a previous paper (Hurtado and Gómez, 2002). FAMEs mass-composition was analyzed by c-GLC (capillary-gas liquid chromatography) using a HP6890 GL chromatograph equipped with an Omegawax 250 fused silica column (30 m x 0.25 mm with 0.25 µm phase from Supelco, Bellefonte, PA). Data were processed electronically and fatty acids were identified on the chromatograms by means of their relative retention times compared with pure fatty acid mixtures processed in parallel (Sigma Chem. Co., Buenos Aires, Argentina). Results were expressed as mol % of total FA.

Free thiol determination

DNA integrity in mature spermatozoa is correlated with the levels of free thiol groups (Zini et al., 2001). Each spermatozoa suspension (8 ml) was centrifuged (600 g; 5 min) and the pellet resuspended in 2 ml of cold TNE buffer (Tris-HCl 0.01 M, pH 7.40 with NaCl 0.15 M and EDTA 1 mM). The suspensions were centrifuged again and resuspended in 500 µl of TNE buffer with 10% glycerol. 200 µl of each final spermatozoa suspension was incubated with 10 µl of SDS 10% (10 min; 25°C), and then treated with 800 µl of DTNB (5,5'-dithiobis(2-nitrobenzoic acid) 0.6 mM in potassium phosphate buffer 0.1 M, pH 7.0 with 6% ethanol). After mixing, the samples were centrifuged at 10000 g for 10 min. The supernatant optical density was measured at 405 nm *vs* blank tubes processed in identical manner but omitting the sample. Thiol concentrations were obtained from a calibration curve using GSH (10 mM in distilled water) as standard solution.

Fructose level

Fructose is produced in seminal vesicles during spermatozoa maturation. Due to its inverse relationship with spermatozoa motility this sugar is considered as a biomarker of seminal vesicle function and motility (Gonzales, 2001). Its concentration was determined in seminal vesicle homogenates (100 µl) after deproteinization with 100 µl of sulfosalicylic acid (10%). Supernatants from centrifugation (14000 rpm; 10 min) were employed for the assay, performed according to Somani et al. (1987). Briefly, 10 µl of each supernatant was incubated (55°C, 90 min) with 1 ml of ATS reagent (10% (w/v) of anthrone and 10% (w/v) tryptophan in sulphuric acid 75% (v/v)) plus 440 µl distilled water. The optical densities were measured at 520 nm against a reaction blank. Fructose concentrations were obtained from a calibration curve using a fructose solution (222 µM in distilled water) as standard.

Other analytical determinations

All plasma hormone measurements (luteinizing (LH), follicle-stimulating (FSH), total and free testosterone (Te), and estradiol) were performed by radioimmunoassay (RIA) using commercial kits (KP7CT, KP6CT, KS24CT, and KS33CTN, respectively) from Radim (Radim SpA, Pomezia, Italy). To measure Te in testicular homogenates, crude preparations were previously centrifuged (2000 g; 15 min) and the supernatants were used as samples for the RIA assay using the kit KS24CT from Radim. Protein content was determined according to Bradford (1976).

Statistical analysis

Results were analyzed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison procedure or Student-t test where appropriate. Data were expressed as the mean ± SD of at least five independent determinations. They were considered different with respect to the control data at two levels of significance $*p < 0.05$ and $**p < 0.01$.

RESULTS

It is well-known that the lipid composition from spermatozoa membranes plays an important role during the fertilization process (Aksoy et al., 2006; Tavilani and Doosti, 2007; Aitken, 1995; Coniglio, 1994). Thus, we studied the fatty acid composition of total lipids from control (C) and treated (ZGD) rats. Figure 1 shows the differences between both groups. Treated rats exhibited a relative decrease of palmitic (16:0) ($p<0.05$) and palmitoleic (16:1) acids content while polyunsaturated fatty acids (PUFAs) 22:4 n-3, 22:5 n-6 and 22:6 n-3 were increased compared to C. Thus, the treated group showed a significant increase in the insaturation index (153.2 ± 18.8 vs 121.4 ± 9.8). PUFAs from both essential series (n-6 and n-3) were elevated in ZGD rats, showing a significant increase in the proportion of n-3 fatty acyl chains compared to control ($p<0.05$). In spite of that, the ratio n-6/n-3 was not significantly different between both groups (5.5 ± 1.2 and 4.0 ± 2.5, respectively) (Table 2).

We also performed morphological studies. The shape and morphology of mature spermatozoa were studied analyzing head, middle zone and tail features. Results were expressed as percentage of total spermatozoa count

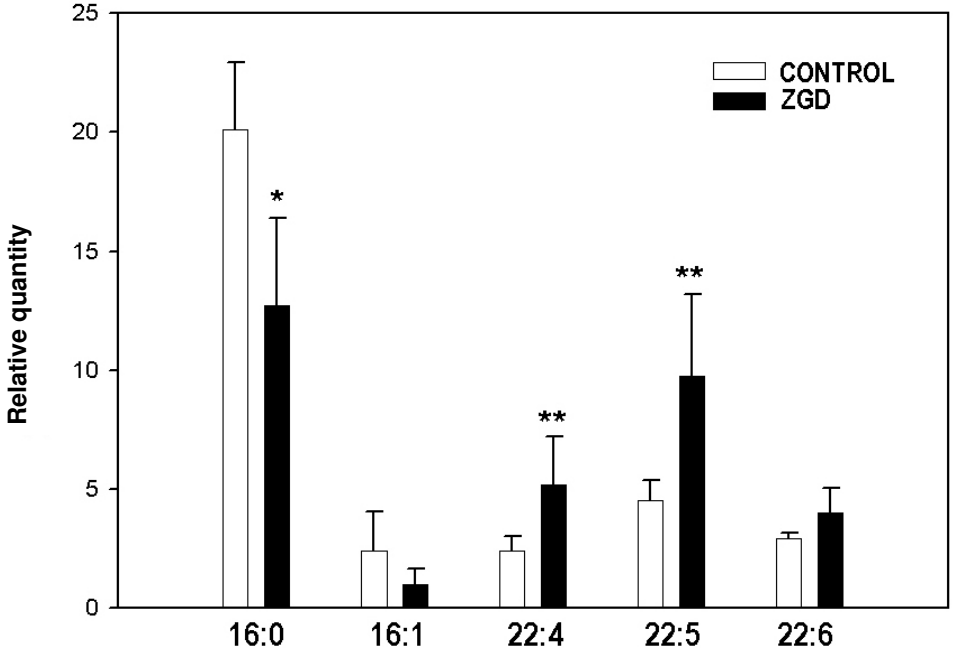

Figure 1. Fatty acid composition of total lipids from epididymal sperm obtained from control (white bars) or (ZGD)-treated (black bars) rats. Animals were treated as described under materials and methods. The figure shows only those fatty acids that showed significant differences compared to controls. Data were expressed as mol percentage. Each bar represents the mean of five independent determinations ± SD. Significant differences from control data were indicated with asterisks (*; $p<0.05$ and **; $p< 0.01$).

Table 2. Fatty-acids composition indexes of epididymal spermatozoa from control and treated rats.

Analytical indexes	Treatment	
	Control	ZGD
Σ Triethilenic fatty acids	2.9 ± 0.7	5.9 ± 2.1 *
Σ Tetraethilenic fatty acids	3.1 ± 0.3	4.9 ± 0.8 **
Σ Pentaethilenic fatty acids	0.9 ± 0.2	10.5 ± 3.5 **
Insaturation index (II)	121.4 ± 9.8	153.2 ± 18.8 *
Σ n-6	33.0 ± 5.7	38.0 ± 1.5
Σ n-3	6.0 ± 0.3	10.0 ± 3.1 *
Σ n-6/Σn-3	5.5 ± 1.2	4.0 ± 0.9

Results were expressed in percentages as the mean ± SD of five independent assays. Values significantly different compared to control group were indicated as *$p< 0.05$ or **$p< 0.01$.

(Figure 2). The exposure to the agrochemical mixture (ZGD) significantly reduced the proportion of spermatozoa with normal morphology (N) with a concomitant increase of cells with morphological aberrations (in MZ, F and in both zones) ($p<0.01$). The agrochemical-induced alterations were similarly distributed among the spermatozoa zones (Figure 2A). Figure 2B is a representative microscopic preparation obtained from a treated rat smear where some evident morphological alterations were observed (arrows). Taking into account that, the evaluation of the spermatozoa membrane integrity is a reliable biomarker of spermatozoa viability currently assessed by staining techniques, we have studied the response of spermatozoa to the eosin-nigrosine (E-N) supra-vital assay. Results obtained are shown in Table 3. Rats treated with the agrochemical

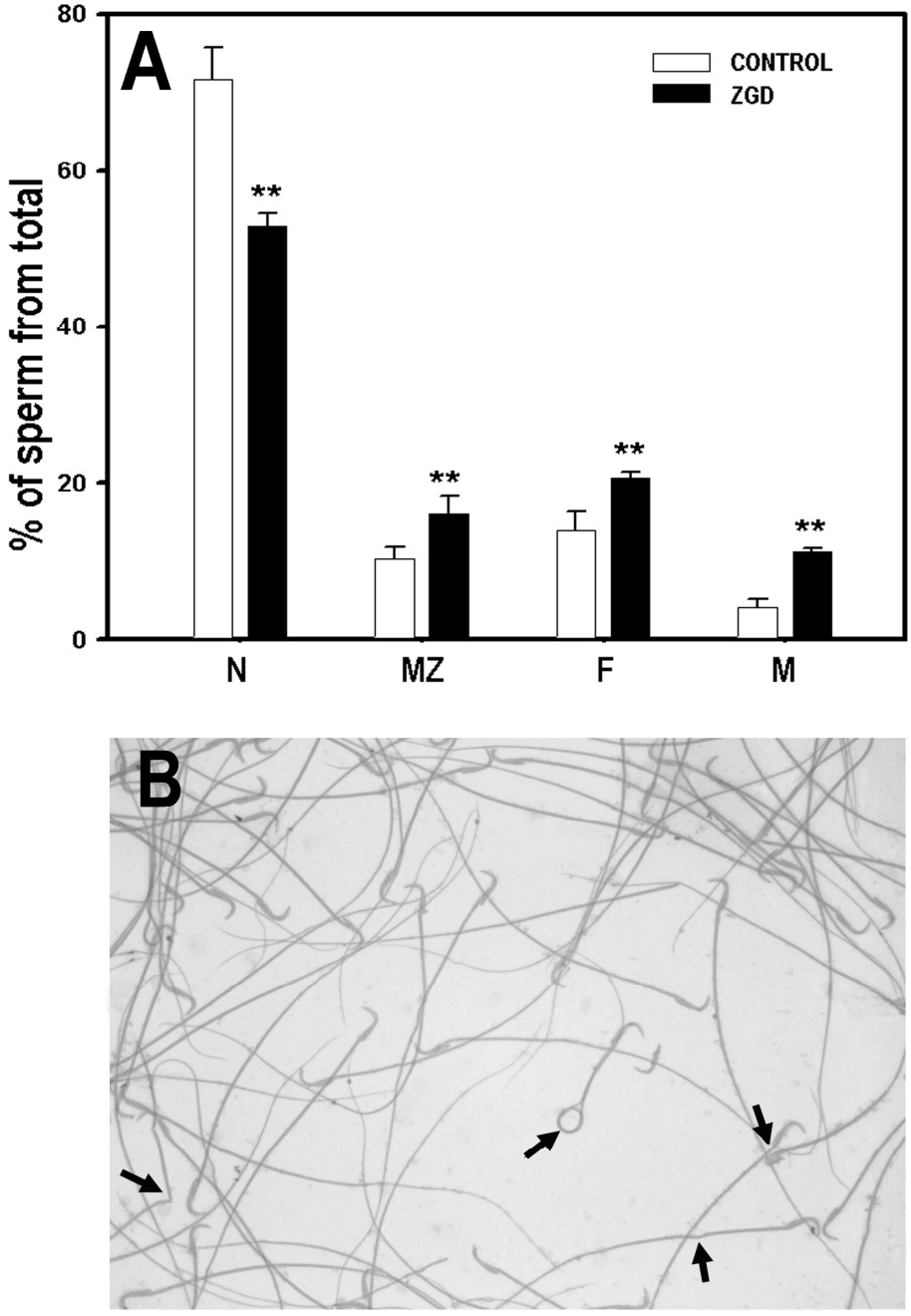

Figure 2. Mature sperm morphology (A) from control (white bars) or (ZGD)-treated (black bars) rats. Animals were treated as described under materials and methods. Briefly, spermatozoa suspension were incubated (25°C, 60 min) with 100 µl of eosin-Y (1% w/v in NaCl 0.9%). Smears were prepared, air dried, covered with Biopack and a cover slip. Five microscopic fields of each slide (approx. 500 cells each) were analyzed using light microscope (400 X). Spermatozoa were classified as follows: N, normal (without alterations); MZ, with altered head and/or middle section; F, flagellum or tail alterations; or M, mixed (combined) alterations. Results were expressed as percentage of the total spermatozoa count following the normal morphology criteria defined by World Health Organization (1999). Each bar represents the mean of five independent determinations ± SD. Significant differences compared to control data were indicated with asterisks (**; $p<0.01$). In panel B a representative microscopic field of a smear from a treated rat, where some evident morphological alterations were indicated (arrows).

Table 3. Functional integrity of sperm cell membrane in control and treated rats.

Condition	Treatment	
	Control	ZGD
Alive	97.85 ± 0.65	95.51 ± 0.33 **
Dying	1.23 ± 0.48	2.31 ± 0.78 **
Died	0.91 ± 0.59	2.17 ± 0.73 **

Results were expressed as the mean ± SD of five independent assays in percentages. Values significantly different compared to control group were indicated with asterisks (**; p< 0.01).

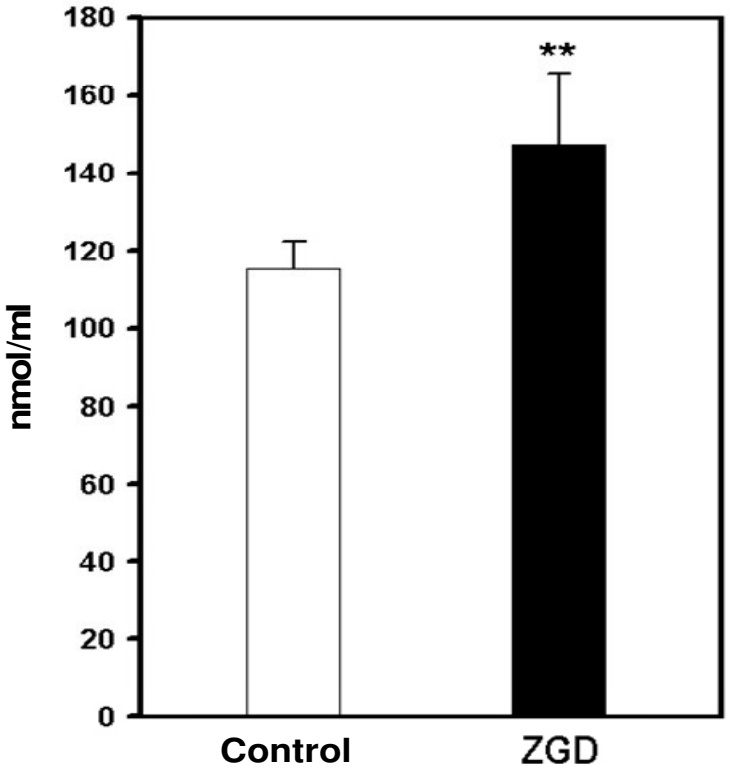

Figure 3. Content of free thiols in mature sperm from caudal epididyms of control (white bar) or (ZGD)-treated (black bar) rats. Animals were treated as described under materials and methods. Data are expressed as nmol/ml. Each bar represents the mean ± SD of five independent determinations. Significant differences from control data were indicated with asterisks (**; p< 0.01).

mixture showed a significant increase of both dying (p<0.01) and dead (p<0.01) spermatozoa, compared to control rats and aconcomitant decrease in the percentage of the living ones (p<0.01).

It is known that the content of spermatozoa free thiols correlates positively with DNA denaturation (Folch et al., 1957). We found that the concentration of this biomarker (expressed as nmol/ml) in epididymal spermatozoa from treated rats was significantly higher compared with that of the control group (p<0.01; Figure 3). Fructose production is usually employed as a marker of seminal vesicle function due to its inverse relationship with spermatozoa motility. Fructose levels in seminal vesicle homogenates were significantly higher in ZGD compared to C rats (p<0.01; Figure 4). Figure 5 shows the percentage of positive acrosome reactions either in control or treated

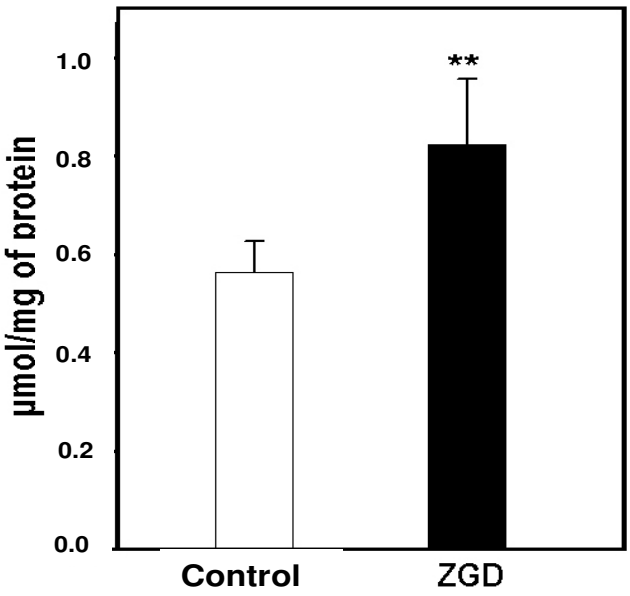

Figure 4. Fructose content in seminal vesicle homogenates from control (white bar) or (ZGD)-treated (black bar) rats. Animals were treated as described under materials and methods. Data were expressed as µmol/mg of protein. Each bar represents the mean ± SD of five independent determinations. Significant differences from control data were indicated with asterisks (**; p< 0.01).

group. The ZGD group showed a significant decrease of spermatozoa with positive acrosome reaction at both times assayed (30 and 60 min) compared to their respective controls. Control spermatozoa reacted in a time-dependent fashion under conditions of pro-activation (Ca^{2+}-ionophore addition). In addition, suppression of Ca^{2+} strongly decreased the percentage of activated spermatozoa at both times. Agrochemical treatment significantly depressed ($p<0.01$) spermatozoa activation (43 and 56% decrease compared to the corresponding control at 30 and 60 min, respectively). Similarly, reacted spermatozoa were strongly reduced compared to control assay under non pro-activating conditions ($p<0.05$). In this case, the reaction time-dependence was completely abolished in both experimental groups (Figure 5).

In order to explore the effects of the agrochemical treatment on the androgenic function, hormonal parameters were determined in both plasma and testis homogenates from control or intoxicated rats using RIA methodologies (Table 4). Free and bound testosterone as well as estradiol were diminished in plasma from treated rats ($p<0.01$), whereas the ratio free/bound Te was indistinguishable between groups. LH and FSH levels in treated rats were significantly increased ($p<0.01$). We also observed a great reduction (approximately 50%) of Te production in testis homogenates obtained from treated rats ($p<0.01$).

DISCUSSION

Several studies suggest that, the decay of human semen quality may be related to the occupational or involuntary exposure to pesticides (Gray et al., 2001; Sanderson, 2006; Recio-Vega et al., 2008; Yucra et al., 2008; Perry, 2008). However, to establish a perfect association with a specific pollutant is almost impossible. More likely, the sum of many environmental contaminants seems to be responsible for the increased incidence of male reproductive dysfunction (Saradha and Mathur, 2006). In addition, the interpretation of experimental results obtained from single intoxication models, often leads to uncertain conclusions mainly associated with the effects displayed by the same pollutant under different contexts (Murono et al., 2001). In this work we used a more realistic animal model sub-chronically exposed to low doses of three of the most frequently used agrochemicals worldwide -administered in combination (ZGD) - to explore the effect of the mixture on semen alterations. This kind of experimental strategy is currently adopted in order to approach the situation in real life, and also to discover and validate robust biomarkers for health risk

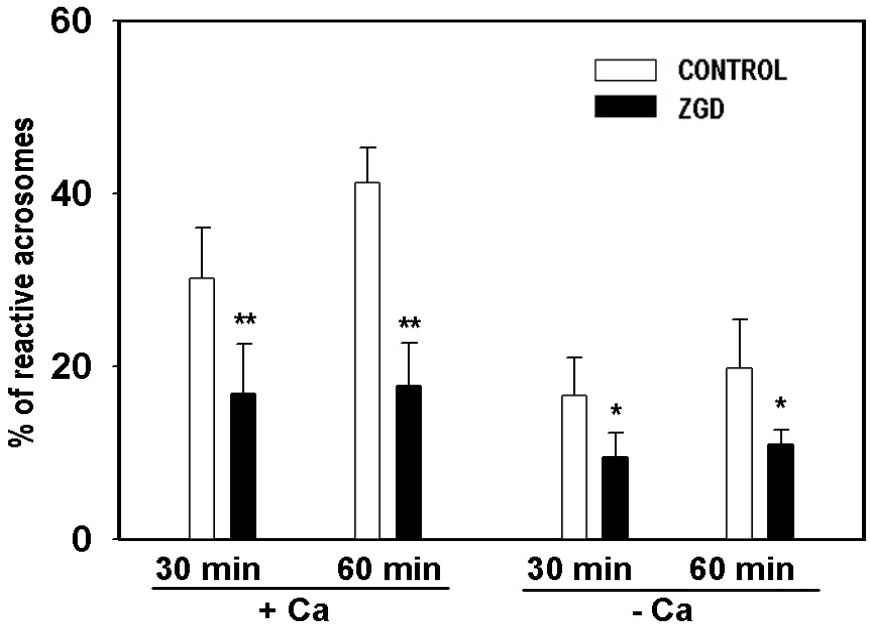

Figure 5. *In vitro* sperm acrosome reaction of control (white bars) and (ZGD)-treated (black bars) rats. Animals were treated as described under materials and methods. Suspensions were incubated in the presence (+ Ca) or in the absence (– Ca) of Ca++ ions for 30 or 60 min at 35°C under gentle shaking. Data were expressed as percentage of reactive acrosomes. Each bar represents the mean ± SD of five independent determinations. Significant differences compared to the corresponding control data were indicated with asterisks (*; $p<0.05$) and **; $p< 0.01$).

assessment (Knudsen and Hansen, 2007).

We have already reported a profound alteration in the redox status of various tissues isolated from rats treated with this agrochemical mixture (Astiz et al., 2009a). Both types of oxidative stress biomarkers - enzymatic and non-enzymatic- were severely altered in plasma and testis indicating a significant unbalance between free radical production and antioxidant defenses. Plasma hormonal levels were also altered. Free and bound testosterone, as well as estradiol levels were depressed in plasma from treated rats while LH and FSH content were increased compared to controls. These results could be interpreted as an adaptative feed-back response of the gonadal-pituitary axis induced by the decay of testosterone level. In line with this fact, we have demonstrated in a recent paper that dimethoate (D) by itself inhibits testosterone biosynthesis in interstitial (Leydig) cells by a mechanism that involves COX-2 and StAR expression, even at the low doses used in our experimental model (Astiz et al., 2009a). The oral administration of technical dimethoate also produces adverse effects on male reproductive performance in mice (Farag et al., 2007).

In addition, two independent reports demonstrated that glyphosate (the active ingredient of Roundup formulation) and octylphenol (a surfactant additive frequently used in many industrial applications) are both inductors of steroidogenic dysfunction (Walsh et al., 2000a; Murono et al., 2000). These results emphasize the importance of performing studies with mixtures of agrochemicals, instead of single-drug experimental models. Traditionally, spermatozoa count is considered as a biomarker of semen quality; however, there are numerous factors that directly or indirectly affect reproduction and reduce the level of desirable statistical power of these results (Seed et al., 1996). In contrast, more recent research determined a direct (positive) relationship between plasma membrane integrity and spermatozoa motility and viability (Dougherty et al., 1975; Vetter et al., 1998; Pesch and Bergmann, 2006). Other authors strongly support the correspondence between oxidative stress biomarkers and spermatozoa characteristics (Abarikwu et al., 2009; El-Taieb et al., 2009), or spermatozoa morphology and genomic integrity of spermatozoa (Zini et al., 2009). All this experimental evidence is in agreement with findings concerning the relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility (Pasqualotto et al., 2000).

Our results clearly indicated that, pesticides provoked

Table 4. Hormonal parameters in plasma and testicular homogenates from control and treated rats.

Determinations	Treatment	
	Control	ZGD
Plasma		
Free testosterone (µM)	4.7 ± 0.2	3.2 ± 0.1**
Bound testosterone (µM)	22.2 ± 1.5	15.3 ± 0.8**
Free/bound testosterone[1]	212 ± 15	209 ± 9
Estradiol (µg/ml)	15.1 ± 0.6	9.3 ± 0.2**
LH (mU/ml)	7.7 ± 0.2	13.8 ± 0.3**
FSH (mU/ml)	8.1 ± 0.4	17.1 ± 0.5**
Testicular homogenates		
Testosterone (µM)	27.9 ± 1.3	11.4 ± 1.5**

Hormone levels were analyzed using RIA kits commercially available from Radim as indicated in Materials and Methods section. Results were expressed as the mean ± standard deviation (SD) of five independent determinations assayed in triplicate. [1][Free/bound testosterone].10^3. Results significantly different respect to control values were indicated with asterisks (**; $p<0.01$).

severe alterations in spermatozoa plasma membrane integrity that could be attributed to a free radical attack. It is well known that epididymal spermatozoa membranes are particularly susceptible to oxidative stress due to their high content of polyunsaturated fatty acids and their lack of Sertoli cell barrier protection (Aitken, 1995). Interestingly, we found that in spite of the pro-oxidant environment induced by pesticide administration the insaturation index of spermatozoa was conserved and even increased compared to controls. This fact is important considering the sub-chronical characteristics of our experimental protocol that may provoke an adaptative response in order to compensate the loss of PUFA from spermatozoa membranes. It is likely that in acute models, the induced oxidative stress may cause peroxidation of PUFAs in the whole testis including the spermatozoa. However, due to the importance of this kind of fatty acyl chains in events associated to fertility performance, chronic exposures may activate a sort of adaptative mechanism (induced biosynthesis, decreased catabolism, selective sequestration, or their combination). Unfortunately, we could not find analytical data reported by other laboratories using a similar experimental condition. Thus, this explanation remains to be further explored.

In line with this, previous work from our laboratory demonstrates that the content of 22:6 n-3 fatty acids in testis from rats under oxidative stress is approximately constant and independent of the pro-oxidant condition (Hurtado de Catalfo et al., 2008; Hurtado de Catalfo et al., 2009). On the other hand, the content of plasmalogens decreases significantly in testis homogenates (Pesch and Bergmann, 2006) but it remains constant in the spermatozoa fraction (Snyder et al, 2001; Nagan and Zoeller, 2001). So, another possible mechanism that justifies the increment in C_{22} PUFAs could be associated to a selective elevation of plasmalogen content in spermatozoa, since this lipid sub fraction is enriched in PUFAs (especially those from the n-3 series) (Coniglio, 1994; Snyder et al., 2001) and it acts as potent anti-oxidant moieties (Snyder et al, 2001; Nagan and Zoeller, 2001). In agreement with this hypothesis we observed a particular increment of the n-3 fatty acyl chains proportion that is intimately associated to the conservation of both the spermatozoa vitality and functionality (Aksoy et al., 2006; Snyder et al., 2001; Furland et a., 2007).

Over the past decades new laboratory tests have been developed to determine properties of spermatozoa function including capacitation, basal and induced acrosome reaction, sperm-zona pellucida interactions and nuclear DNA damage. It was clearly demonstrated that damages to spermatozoa DNA may result in male infertility (Zini et al., 2001; Agarwal and Said, 2003). This could be in part attributed to the reduced ability of mature spermatozoa to repair their own DNA (Van Loon et al., 1991). Our results concerning the levels of spermatozoa protamine free thiol (-SH) groups showed that the combined agrochemical treatment induced a significant increment of these free thiols that correlated with spermatozoa DNA denaturation. This finding is in agreement with those reported by other authors (Zini et al., 2001). Moreover, this biomarker has a positive correlation with the infertility incidence in men (Zini et al, 2001). In addition, it has been recently shown that spermatozoa nuclear and mitochondrial DNA integrity could be a sensitive biomarker of spermatozoa health (Erenpreiss et al., 2006; Lewis et al., 2008). This direct relationship found between free thiols and DNA damage should be considered another putative biomarker with clinical utility for the screening of pesticide-exposed populations.

It is well known that the function of seminal vesicles is under androgen control. Also, it has been demonstrated a direct association between serum testosterone levels, seminal fructose levels, and spermatozoa motility/fertility (Gonzales, 2001). In line with this, we found higher fructose levels in intoxicated rats which could be associated with a poor consumption due to a lower spermatozoa motility induced by agrochemicals. In fact, the epididymal spermatozoa observed under light microscopy, suggested a decreased motility in the treated groups compared to the control one. Epididymal maturation is also an essential process in the transformation of testicular spermatozoa to mature gametes capable of fertilization by the gain of functional

competence (Cooper, 1995). Mature spermatozoa undergo the acrosome reaction when interacting with the zona pellucida of the egg, event that enables it to penetrate the oocyte. Before this binding, spermatozoa cells undergo several biochemical transformations in the female reproductive tract collectively called capacitation. This process involves lipid transfer across plasma membrane (particularly efflux of cholesterol), phospholipids remodeling in plasma membrane (increasing plasma membrane permeability), and changes in protein phosphorylation status, as well as modifications in the intracellular levels of Ca^{2+} and other ions (Abou-haila and Tulsiani, 2009). We have reproduced the acrosomal reaction by an *in vitro* assay, that has been recognized as a predictor of the fertilizing ability of spermatozoa either *in vitro* or *in vivo* (Dematteis et al., 2008). Ca/Ionosphore-induced acrosome reaction was significantly altered in spermatozoa suspensions obtained from treated rats. This assay is easy to be performed as a screening procedure in exposed populations and, as stated before, it should be taken into account as a biomarker of pollutant effects on male reproductive performance.

Results reported in the present study showed that, the mixture of the most frequently used agrochemicals administered at very low doses produced significant detrimental effects on both the spermatozoa characteristics and functional parameters. Therefore, more studies are necessary to attribute the increasing fertility problems as a consequence of the involuntary exposure to low doses of pesticide mixtures over long periods of time. Moreover, we suggested that some of the biomarkers described in this work should be validated and after that, implemented (at least in occupationally exposed populations) in order to establish their cut-off points for their further use in prevention and/or clinical practice.

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