Ecotoxicology and Environmental Safety 91 (2013) 129–138

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Pesticide-induced decrease in rat testicular steroidogenesis is differentially prevented by lipoate and tocopherol

Mariana Astiz [a], Graciela E. Hurtado de Catalfo [a], Marcela N. García [b], Susana M. Galletti [b], Ana L. Errecalde [b], María J.T de Alaniz [a,b], Carlos A. Marra [a,*]

[a] INIBIOLP (Instituto de Investigaciones Bioquímicas de La Plata), CCT La Plata, CONICET-UNLP, Cátedra de Bioquímica y Biología Molecular, Argentina
[b] Cátedra de Histología y Embriología "A", Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120 (1900) La Plata, Argentina

ARTICLE INFO

Article history:
Received 27 October 2012
Received in revised form
17 January 2013
Accepted 21 January 2013
Available online 7 March 2013

Keywords:
Lipoic acid
α-Tocopherol
Rat testis
Oxidative stress
Inflammation
Pesticide mixture
Histological damage

ABSTRACT

We have previously demonstrated that the sub-chronic administration of low doses of Toc or α-Toc, glyphosate and zineb to rats (i.p. 1/250 LD_{50}, three times a week for 5 weeks) provoked severe oxidative stress (OS) in testicles. These effects were also reflected in plasma. Lipoic acid (LA) and α-tocopherol are considered as antioxidants due to their ability to neutralize reactive oxygenated species (ROS) and reset endogenous antioxidant levels. To investigate the possible protective effect on reproductive function, LA and Toc (i.p. 25, 50 and 100 mg/kg) were administered simultaneously with the pesticide mixture (PM) for 5 weeks. Both drugs prevented OS and the damage to proteins and lipids caused by PM in a dose-dependent manner. The PM-induced increase levels of prostaglandins E2 and F2α was completely restored by LA but not by Toc. Similarly, only LA was able to restore the inhibition of testosterone production, the decrease of 3β- and 17β-hydroxysteroid dehydrogenases activities, and the elevation of gonatropins (FSH and LH) levels produced by PM. Furthermore, LA was more efficient than Toc in normalizing the histological alterations produced by PM administration, suggesting that pesticides act though other mechanisms that generate oxidative stress. In our experimental model LA displayed a higher protective role against pesticide-induced damage than that observed by Toc administration. Our results suggest that LA administration is a promising therapeutic strategy for coping with disorders suspected to be caused by OS generators – such as pesticides – in male reproductive system.

1. Introduction

The incidence of male fertility decay has increased alarmingly since 1980s as a result of multi-factorial events including environmental pollution. The number of experimental studies on the effect of agrochemicals on the reproductive system has grown in recent years and some possible mechanisms of toxicity have been suggested. The most widely accepted effect is pesticide-induced hormonal disruption (Sarkar et al., 2000) concomitantly with (or possibly due to) alterations in the antioxidant defense system (Aitken and Barker, 2006; Kesavachandran et al., 2009; Sheweita et al., 2005; Sikka, 2001; Sinclair, 2000).

Humans are exposed to complex mixtures of toxic compounds every day in their residential and occupational environments (Di Monte, 2003; Liu et al., 2003). In particular, agrochemicals are continuously used on a massive scale for global food production and persist as residues in food of both vegetable and animal origin, as well as in the air and water (Bolognesi and Morasso, 2000). Many of the most commonly used pesticides lead to the generation of oxygenated and/or nitrogenated reactive species (ROS/RNS) which affect both the antioxidant levels in mammalian cells and the activity of the scavenging enzyme system (Banerjee et al., 1999; Barlow et al., 2005). Our laboratory studied three of the most commonly used pesticides worldwide: zineb (Z), glyphosate (G), and dimethoate (D), either alone or in combination. Zineb (zinc ethylene-bis-dithiocarbamate) is a widely used contact fungicide (Fitsanakis et al., 2002; Heikkila et al., 1976), glyphosate (*N*-phosphonomethyl-glycine) is a systemic herbicide (Beuret et al., 2005; Daruich et al., 2001) and dimethoate (*O*,*O*-dimethyl-*S*-methyl-carbamoyl-methyl phosphorodithioate) is the most extensively used organophosphorus insecticide of systemic action (Sharma et al., 2005 a,b).

It was previously reported that D impaired sperm motility, decreased serum testosterone levels and testicular weight, and

Non-standard abbreviations: DHLA, dihydrolipoic acid; D, dimethoate; G, glyphosate; LA, α-Lipoic acid; MDA, malondialdehyde; [NOx], nitrate plus nitrite; OS, oxidative stress; PCs, protein carbonyls; PM, pesticide mixture; RNS, reactive nitrogenated species; ROS, reactive oxygenated species; Toc or α-Toc, α-Tocopherol; Z, zineb.

* Corresponding author. INIBIOLP, Cátedra de Bioquímica, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Calles 60 y 120, (1900) La Plata, Argentina. Fax: +54 221 425 8988.
E-mail addresses: camarra@atlas.med.unlp.edu.ar, contactocarlos@hotmail.com (C.A. Marra).

0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.01.022

increased the percentage of dead and abnormal sperm in rats and rabbits (Walsh et al., 2000). In a previous work from our laboratory, we demonstrated that D displays a complex mechanism of action involving disturbance of testosterone production reducing both, the plasma and interstitial Leydig cell concentrations, damage to the antioxidant defense system, decrease araquidonate content in phospholipids, inhibit StAR protein expression with simultaneous stimulation of COX-2 (overproduction of PGF2α) and inhibition of the steroidogenic enzymes 17βHSD and 3βHSD (Astiz et al., 2009a). Damage caused to the steroidogenic status of Leydig cells exposed to D could be reverted by exogenous arachidonate (Astiz et al., 2012). Moreover, recent studies in rats suggested that exposure to G during the pre- and post-natal periods induced adverse effects on male reproductive performance (Dallegrave et al., 2007). An exhaustive bibliographic review by Basrur (2006) showed that G can act as a sexual differentiation disruptor and an estrogen-like compound in domestic animals and humans. Epidemiological evidence indicates that women whose partners have been in contact with G have difficulty conceiving and also show a higher rate of miscarriage (Arbuckle et al., 2001; Caglar and Kolankaya, 2008a,b). In addition, zineb (Z) produced a decrease in mouse fertility due to alterations in male and female pronuclei formation (Rossi et al., 2006). We also demonstrated that a mixture of D, G and Z administered to rats produced both cytological alterations in mature spermatozoa and damage to membrane lipid composition and integrity. Furthermore, the acrosome reaction was impaired and fructose levels incremented in intoxicated rats (Hurtado de Catalfo et al., 2011). The effects found in our works were achieved by very low doses of D, G and Z, since our experimental model aims to simulate the exposure to pesticide residue levels in food.

α-Lipoc acid (LA) is a well-known cofactor of multi-enzymatic complexes which catalyzes the oxidative decarboxylation of α-ketoacids. LA satisfies almost all the criteria of an *ideal antioxidant*, being readily absorbed from the diet and rapidly converted to its redox couple, dihydrolipoic acid (DHLA) in many tissues. Both LA and DHLA effectively quench a number of free radicals ($RO_2^{\cdot}$, HOCl, $O\dot{H}$, ONOOH, $O_2^{\cdot -}$, singlet oxygen) and this antioxidant activity can be displayed in both lipid- and aqueous-cell compartments. They can also chelate redox active transition metals (Fe, Cu), avoiding the production of ROS by the Fenton and Häber–Weiss reactions (Maczurek et al., 2008). Moreover, it is well known that DHLA interacts with other endogenous antioxidants such as glutathione, ascorbate, ubiquinol and indirectly with α-tocopherol (α-Toc), preventing their oxidation (Holmquist et al., 2007; Marangon et al., 1999). α-Toc is a fat-soluble scavenger of peroxyl radicals and probably the most important inhibitor of free-radical chain reaction of lipid peroxidation in mammals (Halliwell and Gutteridge, 1999). It also quenches singlet oxygen, superoxide and peroxide production and protects biological membranes against this species, an effect referred to as *membrane stabilizing* action.

Since exposure to agrochemicals is able to induce OS and there is a clear connection between this condition, inflammation (production of prostaglandins derived from arachidonic acid), and steroidogenesis, we investigated whether the OS condition induced by the administration of low-doses of pesticides could be mitigated (or even reverted) by different doses of LA or Toc. LA may be a good candidate as an antioxidant agent for use in OS-linked diseases in view of the proven scavenger properties (Bilska and Włodek, 2005), and its long-term biosafety (Cremer et al., 2006; Petersen Shay et al., 2009). Similarly, α-tocopherol is well known as the main lipid-soluble antioxidant with a broad range of ROS neutralizing activity (Halliwell and Gutteridge, 1999). In the present work, we explore the impact of the above-mentioned pesticide association on esteroidogenic performance, since pollutants reach body tissue in the form of mixtures present in the environment to which animals and humans are exposed to for prolonged periods (Cory-Slechta, 2005). The aim was to contribute to our knowledge on the protective and/or therapeutic use of LA or α-Toc in relation to the toxic effect(s) caused by environmental pollutants on the male reproductive system.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of reagent grade and were obtained from Sigma Chemical Co. (CA, or Buenos Aires, Argentina) or Merck Laboratories (Darmstadt, Germany). The pesticides employed were zineb (Z; zinc ethane-1,2-diylbis(dithiocarbamate), $C_4H_6N_2S_4Zn$, 99 percent pure, MW: 275.77 g mol^{-1}, CAS number 12122-67-7), dimethoate (D; *O,O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] dithiophosphate, $C_5H_{12}NO_3PS_2$, 98 percent pure, MW: 229.26 g mol^{-1}, CAS number 60-51-5), and glyphosate (G; *N*-[phosphonomethyl]glycine, $C_3H_7NO_5P$, 99 percent pure, MW: 169.09 g mol^{-1}, CAS number 1071-83-6). The drugs were a gift from INTA (Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina).

2.2. Animals and treatments

Male Wistar rats weighing 190 ± 20 g (2 months old) with pathogen-free certified status were used. The rats were maintained under controlled temperature conditions (25 ± 2 °C) and a normal photoperiod of 12 h dark/12 h light, fed on standard Purina chow from Ganave Co. (Santa Fe, Argentina) and given water *ad libitum*. Clinical examination and body weights evaluation were performed every week during the experiment. The animals were randomly divided into groups of six.

We evaluated the possible protective effect of LA or Toc administered intraperitoneally (i.p.) in three different doses (25, 50 and 100 mg/kg body weight (bw)), the animals were treated with the antioxidants five times a week for five weeks. Simultaneously, the animals were treated with the pesticide mixture (PM) or vehicle three times a week for five weeks. The pesticide mixture (PM) consist of Z (15 mg/kg bw) plus G (10 mg/kg bw) plus D (15 mg/kg bw), all injected i.p. and dissolved in the same vehicle (polyethylene-glycol 400, PEG-400). The study included the following experimental groups: C (treated i.p. with PEG-400); T (treated i.p. with PM); CLA25, CLA50 and CLA100 (treated i.p. with LA, 25, 50 or 100 mg/kg bw, respectively); TLA25, TLA50 and TLA100 (treated i.p. with PM and simultaneously with 25, 50 or 100 mg LA/kg bw); CToc25, CToc50 and CToc100 (treated i.p. with Toc, 25, 50 or 100 mg/kg bw, respectively); and TToc25, TToc50 and TToc100 (treated i.p. with PM and simultaneously with 25, 50 or 100 mg Toc/kg bw).

Our treatment schedule (time and doses) was designed on the basis of our own previous experimental protocols and those of other laboratories (Arivazhagan et al., 2000; Astiz et al., 2009b,c,d; Bagchi et al., 1995; Beuret et al., 2005; Cremer et al., 2006; Daruich et al., 2001; Fitsanakis et al., 2002; John et al., 2001; Nagamatsu et al., 1995; Sharma et al., 2005a,b; Tang et al., 2007).

Animal maintenance and handling procedures were in accordance with the NIH guide for the care and use of laboratory animals (National Research Council, 1985). All the experiments were approved by the local Laboratory Animal Bioethics Committee, Facultad de Ciencias Médicas, UNLP, Argentina (protocol COBIMED #001241/10). The model employed was developed to simulate subchronic exposure to low doses of pesticide mixtures such as those incorporated from the general environment (Cory-Slechta, 2005). The animals were observed for behavioral performance and symptoms of possible intoxication. A veterinary doctor evaluated the presence of miosis, mouth smacking, salivation or lacrimation and the rats were also placed in an open field for observation of tremors and gait abnormalities (Moser et al., 2006). There was no significant evidence of differences between control and treated animals. Body weight gains and absolute testis and testis-to-body weights (testis relative weight) were determined for each experimental group.

2.3. Sample collection

At the end of the treatments (5 weeks), animals were killed by rapid decapitation. Blood was collected using heparin as anticoagulant in ice-cold polypropylene tubes. Plasma was immediately prepared by centrifugation (4000 × *g*, 10 min) and then stored at −80 °C. Testes were excised, washed, weighed, and quickly homogenized in ice-cold phosphate buffer 140 mM pH 7.40, with 6 mM of EDTA, in a ratio of 5 ml buffer to 1 g of tissue. Samples were stored at −80 °C under nitrogen atmosphere to avoid peroxidation. Representative samples of both testes were taken for histological studies and immediately fixed in paraformaldehyde (10 percent in PBS pH 7.40).

2.4. Analytical methods

2.4.1. Biomarkers of damage

Thiobarbituric acid reactive substances (TBARS) and protein carbonyls (PCs) were used as biomarkers of pro-oxidative damage to lipids and proteins, respectively (Dalle-Donne et al., 2006; Halliwell and Gutteridge, 1999). Lipid peroxidation (LPO) was assayed using the TBARS assay with tetraethoxypropane as standard by the method of Yagi (1976). PCs were determined using the method described by Reznick and Packer (1994). The sum of nitrates and nitrites [NO*x*] was determined as the main end-metabolite of nitric oxide (NO) and peroxinitrite anion ($ONOO^-$) using samples previously deproteinized, reduced with VCl_3, and then subjected to the Griess reaction according to Miranda et al. (2001). Briefly, appropriate aliquots of samples were treated with four volumes of a methanol:-chloroform mixture (3:1) at 4 °C. After centrifugation at 10,000 *g* for 10 min, they were reduced by an excess of VCl_3 in 1 M HCl. Samples were combined with the Griess detection reactive (*N*-1-(naphthyl) ethylenediamine (1 mM) plus sulfamide (20 mM) in 5 percent HCl. The formation of the chromophore from the diazotization of sulfamide by nitrite at low pH, followed by the coupling with the bicyclic amine, was detected at 405 nm after incubating the mixtures at 37 °C for 30 min. Quantification was performed after calibration with standard solutions of sodium nitrate from Merck Co. (Darmstadt, Germany).

2.4.2. Antioxidants

2.4.2.1. α-Tocopherol (Toc). We measured vitamin E (α-tocopherol, α-Toc) as a non-enzymatic biomarker of the antioxidant defense system within the lipid-soluble cell compartment. Toc was determined after extraction by the method of Buttriss and Diplock (1984) using the HPLC technique of Bagnati et al. (1998).

2.4.2.2. Free thiol (FT) determination. Free thiols, as one of the most important contributors to the antioxidant status of the water-soluble cell compartment, were determined following the methodology described elsewhere (Zini et al., 2001a,b). Each sample (200 µl) was incubated with 2 ml of cold TNE buffer (Tris–HCl 0.01 M, pH 7.40 with NaCl 0.15 M and EDTA 1 mM) with the addition of 100 µl of SDS 10 percent (10 min; 25 °C). Then they were treated with 800 µl of DTNB (5,5′-dithiobis(2-nitrobenzoic acid) solution (0.6 mM in potassium phosphate buffer 0.1 M, pH 7.0 with 6 percent ethanol). After mixing, the samples were incubated again (10 min; 25 °C) and centrifuged at 10,000 *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.

2.4.2.3. Ferric reducing ability of plasma (FRAP) assay. The FRAP assay (Ferric Reducing Ability of Plasma) used to assess the whole antioxidant ability of samples, was determined by the method of Benzie and Strain (1996) and expressed as mmol of equivalent Trolox® or α-tocopheryl diacetate/mg of protein. The samples were previously treated with uricase dissolved in phosphate buffer 50 mM/EDTA 1 mM/glycerol 25 mM (pH 8.50) in a proportion of 1 IU enzyme/100 µL sample to avoid the contribution of uric acid to the total antioxidant capacity. Aliquots (100 µl) were incubated in 3 ml of acetic acid/acetate buffer (300 mM, pH 3.60) containing 100 µM TPTZ (tripyridil-triacine) and $FeCl_3$ (50 mM). The formation of the complex [Fe(II)–TPTZ] depends of the antioxidant capacity of the mixture and can be followed at 583 nm. The slope of the (linear) kinetic profile obtained after the first minute of reaction is proportional to the formation of ferrous cation and can be compared with a standard solution of Trolox®.

2.4.3. Hormone measurements

Plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH) and total testosterone measurements were performed by radioimmunoassay (RIA) using commercial kits (KP7CT, KP6CT, and KS24CT, respectively) from Radim (Radim SpA, Pomezia, Italy). To measure testosterone in testicular homogenates, crude preparations were first centrifuged (20,000 *g* for 20 min at 2 °C) and the supernatants used as samples in an RIA assay using the KS24CT Radim kit. Protein content was determined according to the method of Bradford (1976).

2.4.4. Prostaglandin (PG) determination

$PGF_{2\alpha}$ and PGE_2 were determined in testis homogenates. The samples were centrifuged at 15,000 *g* for 30 min at 2 °C. Supernatants were filtered though Millipore 0.45 µm membranes and concentrated by liophilization in a Telstar apparatus, Lyobeta Model (Madrid, Spain). The residues were dissolved in HCl 2 N, adjusting the pH to 3.5, injected into a 200-mg C_{18} reverse phase Sep-column (Peninsula Lab, Belmont, CA), and then eluted with ethyl acetate. The eluted fractions were evaporated to dryness under a nitrogen stream and reconstituted in buffers for enzyme-immuno assay (EIA) determinations. PGs were determined using commercially available kits (Prostaglandin $F_{2\alpha}$ EIA Kit and Prostaglandin E_2 Express EIA Kit from Cayman, Migliore Laclaustra S.R.L. (Buenos Aires, Argentina) with a minimum detection of 4 and 30 pg/ml, respectively. Intra- and inter-assay coefficients of variation were in the range of 8–12 percent for both kits. Results were expressed as ng/mg protein.

2.4.5. Steroidogenic enzyme activities

Appropriate aliquots of homogenate supernatants were employed to determine 3β-hydroxysteroid-dehydrogenase (3β HSD) and 17-β-hydroxysteroid-dehydrogenase (17β HSD) [EC 1.1.1.51] enzyme activities following the method of Murugesan et al. (2005). In brief, the samples were centrifuged (12,000 × *g*, 15 min, 1–2 °C) and the supernatants were mixed with charcoal in order to remove the endogenous steroids. The mixtures were again centrifuged (12,000 × *g* 15 min, 2 °C). The reaction conditions were defined in preliminary experiments in which enzyme activities were tested by varying the incubation times and concentrations of substrates, protein, and cofactors. Assays were performed under initial velocity conditions in a reaction mixture (250 µl final volume) containing 10 µM of the steroid substrate (Δ^4-androstenedione or pregnenolone for 17β HSD and 3β HSD, respectively), 1 mM NADPH (17βHSD) or 0.2 mM NAD^+ (3β HSD), and 0.1 M phosphate buffer (pH 7.4). Mixtures were incubated under air in a spectrophotometric cell holder thermostatized at 37 °C. The reactions were started by the addition of the supernatant aliquots and OD changes of the nicotinamide cofactor(s) were measured. The incubation mixture deprived of substrate(s) was used as reagent blank.

2.5. Histological studies

Representative portions of testis were taken and fixed in PBS-paraformaldehyde (10 percent), pH: 7.40), sequentially dehydrated, included in paraffin, sectioned (5 µm) using a Reichter 2040 Microtome (Medical Equipment Source, LLC; PA, USA), and processed following the conventional hematoxylin–eosin methodology. Slides were mounted on synthetic Canada balsam (BioPur, Rosario, Argentina) and examined (three slides per rat, at least three different fields each) by the same histopathologist under light microscopy. Histological damage was classified according to the procedure described by Urdaneta de Romero et al. (1998) in seven degrees of involution/degeneration (being 0 assigned to normal tubules and germinal epithelium, and 2–7 to different degrees of damage).

2.6. Statistical analysis

All values represent the mean of six rats analyzed in duplicate and are expressed as the mean ± standard deviation (SD). Data were analyzed using one way ANOVA followed by Tukey's *post hoc* test with the aid of Systat (version 15.0 for Windows) from SPSS Science (Chicago, IL). Statistically significant differences at the level $p \leq 0.01$ were indicated by different letters.

3. Results

3.1. Effects of LA administration on body weights

The groups treated with 100 mg/kg bw of LA showed a significant decrease in body weight gain, final body weight, and food efficiency ratios (data not shown). There was a drop of approximately sixteen percent in the weight gain curve that became statistically significant from day 14 and persisted up to the end of the experimental period. The administration of 25 or 50 mg LA/kg bw gave rise to lower decreases in body weight gain which were not statistically significant compared to controls. Absolute and relative testis weights were indistinguishable among experimental groups.

3.2. PM-induced modification of OS biomarkers

The degree of damage to lipids and proteins induced by the sub-chronic administration of the PM is shown in Table 1. The PM provoked significant oxidative damage to proteins and lipids from testis homogenates. This was reflected in peripheral plasma. Furthermore, a substantial degree of nitrosative stress was observed as indicated by the elevation in both the local and systemic concentrations of [NO*x*]. The co-administration of PM and LA produced a dose-dependent reduction of these three biomarkers, achieving basal values with the medium or higher dose of LA administered. In addition, control rats treated with LA showed a reduction in basal protein damage and lipid peroxidation. These

Table 1
Biomarkers of oxidative damage in plasma and in testis homogenates prepared from rats treated with the pesticide mixture and/or α-lipoic acid (LA) or α-tocopherol (Toc).

Groups	Plasma			Homogenates		
	PCs	TBARS	[NO*x*]	PCs	TBARS	[NO*x*]
C	3.02 ± 0.08^{a}	2.22 ± 0.13^{a}	0.61 ± 0.04^{a}	2.15 ± 0.07^{a}	1.44 ± 0.04^{a}	10.61 ± 0.08^{a}
T	5.55 ± 0.12^{b}	3.42 ± 0.11^{b}	1.08 ± 0.07^{b}	6.01 ± 0.22^{b}	1.98 ± 0.04^{b}	23.5 ± 0.10^{b}
LA						
CLA25	2.80 ± 0.08^{a}	2.01 ± 0.10^{a}	0.60 ± 0.12^{a}	2.03 ± 0.11^{a}	1.38 ± 0.05^{a}	11.51 ± 0.15^{a}
CLA50	2.36 ± 0.12^{c}	2.16 ± 0.05^{a}	0.51 ± 0.15^{a}	1.77 ± 0.04^{c}	1.30 ± 0.11^{a}	10.43 ± 0.12^{a}
CLA100	2.40 ± 0.05^{c}	1.48 ± 0.10^{c}	0.40 ± 0.04^{b}	1.66 ± 0.11^{c}	0.81 ± 0.06^{c}	9.09 ± 0.11^{a}
TLA25	4.42 ± 0.15^{d}	2.30 ± 0.14^{a}	0.81 ± 0.05^{c}	2.90 ± 0.22^{d}	1.59 ± 0.15^{d}	15.11 ± 0.09^{c}
TLA50	3.11 ± 0.12^{a}	2.27 ± 0.13^{a}	0.63 ± 0.04^{a}	2.30 ± 0.05^{e}	1.45 ± 0.10^{a}	12.05 ± 0.11^{a}
TLA100	3.05 ± 0.18^{a}	1.69 ± 0.04^{d}	0.58 ± 0.05^{a}	2.15 ± 0.11^{a}	1.08 ± 0.09^{e}	12.40 ± 0.16^{a}
Toc						
CToc25	2.86 ± 0.11^{a}	2.03 ± 0.14^{a}	0.66 ± 0.10^{a}	3.88 ± 0.13^{b}	0.80 ± 0.05^{c}	11.22 ± 0.17^{a}
CToc50	2.41 ± 0.08^{c}	1.13 ± 0.10^{e}	0.59 ± 0.05^{a}	3.72 ± 0.14^{b}	0.78 ± 0.10^{c}	10.38 ± 0.22^{a}
CToc100	2.10 ± 0.12^{d}	1.05 ± 0.09^{e}	0.51 ± 0.14^{a}	3.61 ± 0.20^{b}	0.80 ± 0.20^{c}	10.10 ± 0.14^{a}
TToc25	4.05 ± 0.06^{a}	1.60 ± 0.08^{d}	1.01 ± 0.13^{b}	4.41 ± 0.22^{a}	1.32 ± 0.15^{a}	20.15 ± 0.14^{b}
TToc50	3.20 ± 0.15^{a}	1.40 ± 0.11^{f}	0.99 ± 0.10^{b}	4.30 ± 0.15^{a}	1.10 ± 0.16^{a}	19.85 ± 0.31^{b}
TToc100	3.16 ± 0.07^{a}	1.31 ± 0.10^{f}	0.65 ± 0.14^{a}	4.18 ± 0.21^{a}	0.86 ± 0.09^{c}	13.01 ± 0.22^{a}

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in the Material and methods section. PCs, TBARS and [NO*x*] were expressed in μmol pyruvate/mg protein, nmol MDA/mg protein; μmol nitrite/mg protein; respectively. Results statistically different within the same column are indicated with distinct superscript letters (ANOVA+Tukey test; $p < 0.01$). C, representative data for control rats injected with saline solution, or the vehicles used to dissolve LA or Toc (no statistically different among them); T, group treated with the pesticide mixture alone; CL, treated with LA; TL and TToc, treated simultaneously with pesticide mixture and LA or α-tocopherol, respectively.

Table 2
Biomarkers of hormonal and pro-inflammatory damage in plasma isolated from rats treated with the pesticide mixture and/or α-lipoic acid (LA) or α-tocopherol (Toc).

Groups	Testosterone (pM)	FSH (mIU/ml)	LH (mIU/ml)	PGE2 (ng/mg protein)	PGF2α (ng/mg protein)
C	24.72 ± 4.25^{a}	10.66 ± 0.30^{a}	12.11 ± 0.56^{a}	38.87 ± 1.50^{a}	23.45 ± 1.82^{a}
T	13.31 ± 1.22^{b}	16.97 ± 0.55^{b}	23.25 ± 1.88^{b}	62.64 ± 2.51^{b}	35.19 ± 1.54^{b}
LA					
CLA25	25.13 ± 2.93^{a}	11.01 ± 0.56^{a}	12.44 ± 0.72^{a}	33.90 ± 1.41^{c}	22.64 ± 1.71^{a}
CLA50	23.39 ± 3.03^{a}	10.83 ± 0.61^{a}	11.58 ± 0.66^{a}	30.56 ± 2.42^{c}	18.08 ± 1.11^{c}
CLA100	24.90 ± 1.89^{a}	11.50 ± 0.77^{a}	12.22 ± 0.49^{a}	25.15 ± 2.04^{d}	16.55 ± 1.62^{c}
TLA25	17.22 ± 2.94^{c}	13.40 ± 0.45^{c}	12.27 ± 0.61^{a}	44.11 ± 1.71^{e}	30.12 ± 1.73^{d}
TLA50	24.44 ± 2.31^{a}	11.01 ± 0.80^{a}	11.06 ± 0.37^{a}	34.20 ± 1.37^{c}	24.14 ± 1.60^{a}
TLA100	23.87 ± 2.07^{a}	10.85 ± 0.53^{a}	10.74 ± 0.77^{a}	33.10 ± 1.55^{c}	22.78 ± 1.91^{a}
Toc					
CToc25	24.53 ± 2.0^{a}	11.08 ± 1.22^{a}	10.89 ± 1.15^{a}	37.06 ± 2.22^{a}	22.57 ± 1.76^{a}
CToc50	23.118 ± 1.9^{a}	11.43 ± 2.50^{a}	12.51 ± 0.85^{a}	36.90 ± 1.75^{a}	23.13 ± 1.99^{a}
CToc100	24.14 ± 1.6^{a}	10.35 ± 1.94^{a}	11.33 ± 0.77^{a}	38.41 ± 1.20^{a}	22.85 ± 2.12^{a}
TToc25	14.33 ± 2.2^{a}	16.16 ± 1.55^{b}	22.91 ± 1.01^{b}	64.01 ± 2.33^{b}	36.02 ± 1.83^{b}
TToc50	14.58 ± 3.1^{a}	15.81 ± 2.44^{b}	23.43 ± 0.77^{b}	62.47 ± 1.55^{b}	35.41 ± 1.66^{b}
TToc100	15.82 ± 3.3^{a}	16.02 ± 2.13^{b}	23.05 ± 0.81^{b}	63.24 ± 2.31^{b}	34.47 ± 2.09^{b}

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in the Material and methods section. Results statistically different within the same column are indicated with distinct superscript letters (ANOVA+Tukey test; $p < 0.01$). C, representative data for control rats injected with saline solution, or the vehicles used to dissolve LA or Toc (no statistically different among them); T, group treated with the pesticide mixture alone; CL, treated with LA; TL and TToc, treated simultaneously with pesticide mixture and LA or α-tocopherol, respectively.

protective effects were of lesser magnitude in the case of [NO*x*]. Simultaneous administration of PM and Toc demonstrated a similar pattern of restoration of PC levels to that observed for LA; however, Toc, a antioxidant which is directly correlated to lipid peroxidative damage protection, appears more efficient than LA in neutralizing TBARS levels. Toc was able to decrease [NO*x*] concentration in plasma or testis only at the higher dose assayed (Table 1).

3.3. Hormonal levels in treated animals

The treatment with PM produced a marked decrease in the plasma testosterone level (approx. 54 percent compared to control) and a concomitant increase in the concentration of the main gonadotropin hormones, FSH and LH, which were 54 and 90 percent higher, respectively, than basal data (Table 2). LA or Toc injection to control animals did not introduce any significant changes in the (basal) hormonal levels studied. Co-administration of PM with LA restored the level of testosterone at doses of 50 or 100 mg/kg bw. Furthermore, overproduction of both FSH and LH was normalized (Table 2). In contrast, Toc – administered at any dose assayed – was not able to restore or even attenuate the PM-induced alterations observed in the hormonal levels (Table 2).

3.4. Biomarkers of inflammation

The treatment with PM also produced a significant inflammatory response reflected in plasma by an almost twofold increase in the levels of both PGE_2 and $PGF_{2\alpha}$ (Table 2) compared with control data. The administration of LA alone tended to decrease the concentration of both prostaglandins in a dose-dependent manner, an effect that was not observed with Toc. Supplementation of PM with LA completely restored the levels of PGE_2 and $F_{2\alpha}$ (and even decreased them below control data), while Toc co-administration was completely ineffective (Table 2).

3.5. Androgen biosynthesis in treated animals

The androgenic status was in agreement with the activities of the main enzymes involved in testosterone biosynthesis, 3β–HSD and 17β–HSD. Both enzymes catalyze key steps in the androgenic pathway and are under the influence of environmental pollutants (Astiz et al., 2011, 2012). In fact, both enzymes decreased sharply under PM administration (50 and 31 percent with respect to basal data, respectively) (Table 3). LA administration to control rats produced no significant effects on 3β–HSD activity but a slight increment – at the medium or higher dose – on the 17β–HSD. Toc injection did not substantially modify the activity of the enzymes. The co-administration of PM with LA normalized the activities of 3β- and 17β–HSD in a clear dose-dependent manner, while Toc restored the activities at the higher dose assayed (Table 3).

3.6. Antioxidants levels in treated rats

The administration of Toc to control rats was reflected in the level of the vitamin in testis in a dose-dependent fashion (29, 43 and 71 percent increments over basal data, respectively). Interestingly, the higher amount of LA administered to control rats produced a 29 percent increase in the level of α-Toc in testis, equivalent to that observed with the lowest dose of the vitamin injected. The PM-induced OS caused a significant consumption of α-Toc that led to a reduction of almost 57 percent in the level of this vitamin in testis homogenates (Table 3). Co-administration of PM with LA attenuated this effect at the lowest dose assayed and normalized the concentration at 50 or 100 mg/kg bw. Under the effects produced by PM, the co-administration of Toc only maintains the level of the vitamin close to that of control rats (Table 3). The FRAP assay reflects the balance between all the substances present in tissue homogenates (with the exception of uric acid) with pro- or anti-oxidant activities. Clearly, PM administration leads to a reduction in FRAP values that is completely reverted by LA or Toc co-administration. Toc was more efficient than LA in increasing FRAP values in control rats (Table 2). PM treatment led to an almost 62 percent decrease in total free thiol content. LA administration almost restored FT to normal values partially at the lowest dose assayed and completely at higher doses, FT levels were completely normalized. Toc did not modify the concentration of FT in control rats and failed to restore FT in PM-treated animals at any dose assayed (Table 3). In contrast, LA administered to control rats produced a dose-dependent increase in FT values (10, 21 and 30 percent over control data, respectively).

3.7. Histological studies

Histological studies (Fig. 1) revealed that PM was able to induce severe damage to tissue architecture involving both the germinal cell series and interstitial (Leydig) cells. Treated animals showed a significant loss of germ cells (hypospermatogonia) with decay in the production of mature spermatozoa (visible in the interior of seminiferous tubules), decreased Leydig cells in the interstitial space with the presence of giant and picnotic cells (damages varied from five to seven according to the scale of Urdaneta de Romero et al. (1998)), contrasting with the normal arrangement shown by control rats (score 0–1). Unfolding of the basal membranes was also observed. Combined treatment with PM and LA (at a dose of 50 mg/kg bw) showed nearly normal architecture of seminiferous tubules and interstitial cells (score 1–2), while co-treatment with PM and Toc (at a dose of 50 mg/kg bw) partially restored the histological architecture to normal (score 2–4) decreasing the alterations induced by PM treatment.

4. Discussion

Our results show that PM exposure provoked a marked OS condition, increasing biomarkers of damage to lipids and proteins in whole testis homogenates. The OS is also observed in peripheral plasma. Furthermore, we found a pro-inflammatory state and an abnormal steroidogenic balance with androgen depletion and over-stimulation by gonadotrophins together with the inhibition of 3β- and 17β–HSD activities. These results corroborate previous findings from our laboratory (Astiz et al., 2009a,b; Hurtado de Catalfo et al., 2011). LA treatment caused a general improvement in this condition, which was only partially prevented by Toc administration. We observed a significant loss of weight gain in those groups receiving 100 mg/kg bw LA. This could be due to the anti-obesity effect of LA previously documented by Cremer et al. (2006) and Kim et al. (2004) and/or other unknown effect(s), all of which should be taken into consideration when evaluating the protective properties of this drug in terms of the dose to be administered.

Table 3
Steroidogenic activities and redox status in homogenates prepared from rats treated with the pesticide mixture and/or α-lipoic acid (LA) or α-tocopherol (Toc).

Groups	3β–HSD (nmol min g protein)	17β–HSD (nmol min mg protein)	Toc (nmol/μg protein)	FRAP (mmol/mg protein)	FT (μmol/mg protein)
C	2.19 ± 0.05^{a}	0.45 ± 0.04^{a}	0.14 ± 0.02^{a}	11.61 ± 0.70^{a}	55.25 ± 1.63^{a}
T	1.10 ± 0.08^{b}	0.14 ± 0.02^{b}	0.08 ± 0.02^{b}	7.12 ± 0.21^{b}	33.82 ± 1.89^{b}
LA					
CLA25	2.03 ± 0.11^{a}	0.43 ± 0.03^{a}	0.13 ± 0.04^{a}	11.85 ± 0.43^{a}	57.22 ± 1.44^{a}
CLA50	2.16 ± 0.08^{a}	0.53 ± 0.02^{c}	0.14 ± 0.02^{a}	12.66 ± 0.50^{a}	66.31 ± 1.58^{c}
CLA100	2.22 ± 0.05^{a}	0.55 ± 0.03^{c}	0.18 ± 0.03^{c}	13.87 ± 0.44^{c}	72.43 ± 2.0^{d}
TLA25	1.55 ± 0.08^{c}	0.34 ± 0.04^{d}	0.10 ± 0.02^{d}	10.02 ± 0.52^{a}	42.52 ± 1.77^{e}
TLA50	2.05 ± 0.11^{a}	0.46 ± 0.10^{a}	0.13 ± 0.05^{a}	10.30 ± 0.45^{a}	57.35 ± 1.60^{a}
TLA100	2.12 ± 0.21^{a}	0.45 ± 0.04^{a}	0.13 ± 0.03^{a}	12.12 ± 0.51^{a}	58.84 ± 2.11^{a}
Toc					
CToc25	2.23 ± 0.11^{a}	0.48 ± 0.11^{a}	0.18 ± 0.05^{c}	13.11 ± 0.55^{a}	54.44 ± 1.55^{a}
CToc50	2.36 ± 0.11^{a}	0.44 ± 0.05^{a}	0.20 ± 0.03^{c}	16.42 ± 0.61^{d}	55.12 ± 2.10^{a}
CToc100	2.20 ± 0.05^{a}	0.45 ± 0.11^{a}	0.24 ± 0.04^{e}	18.11 ± 0.52^{d}	53.27 ± 1.86^{a}
TToc25	1.22 ± 0.08^{b}	0.15 ± 0.02^{b}	0.12 ± 0.05^{a}	10.20 ± 0.32^{a}	34.52 ± 1.51^{b}
TToc50	1.18 ± 0.11^{b}	0.16 ± 0.08^{b}	0.14 ± 0.02^{a}	11.33 ± 0.65^{a}	35.04 ± 2.66^{b}
TToc100	1.38 ± 0.05^{c}	0.35 ± 0.04^{c}	0.15 ± 0.03^{a}	12.15 ± 0.41^{a}	35.15 ± 1.79^{b}

Results were the mean of six animals assayed in duplicate ± SD. Samples were processed as detailed in the Material and methods section. Results statistically different within the same column are indicated with distinct superscript letters (ANOVA+Tukey test; $p < 0.01$). C, representative data for control rats injected with saline solution, or the vehicles used to dissolve LA or Toc (no statistically different among them); T, group treated with the pesticide mixture alone; CL, treated with LA; TL and TToc, treated simultaneously with pesticide mixture and LA or α-tocopherol, respectively.

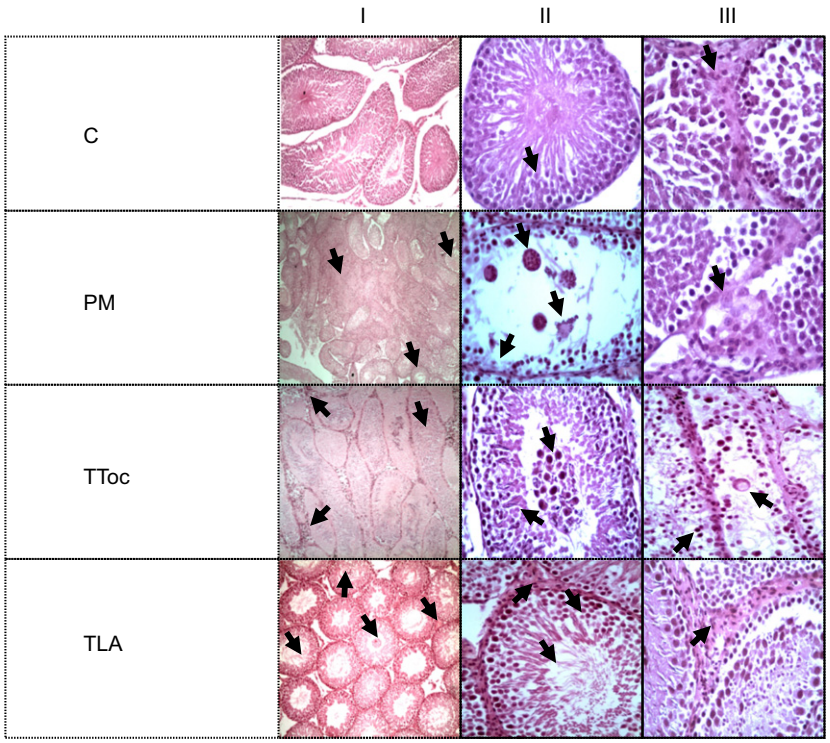

Fig. 1. Histological examination (haematoxylin-eosin methodology) after the experimental treatments. Representative images from C, control group; PM, pesticide mixture-treated rats; TToc, group treated with PM and Toc (50 mg/kg bw); TLA, group treated with PM and LA (50 mg/kg bw). (I) Panoramic (10 ×) views, (II) seminiferous tubules details (40 ×), (III) interstitial space (40 ×). Arrows indicate major findings. C-I, II, III shows normal panoramic, tubular, and interstitial histology. Spermatozoa and typical interstitial (Leydig) cells are clearly visible. PM-I shows a complete loss of tubular architecture and disappearance of the interstitial space. Only some relatively conserved tubules with a rudimentary interstitial space were seen. In exceptional zones the presence of intra-luminal spermatozoa was also observed. PM-II, III shows abundant giant multinucleate cells and cell debris with a concomitant loss of the germinal series and disorganization of the interstitial space. Toc-I showed a partial reorganization of tubular architecture and interstitial space. Toc-II shows almost complete loss of the germ cell series and the presence of intra-luminal giant cells and cellular debris. Although not so clear, the histological scenario was similar to that founded in PM-II. Toc-III also shows giant multi-nucleate cells within the seminiferous tubules with a complete loss of the cells that constitute the germinal series. Scare and damage Leydig cells in the interstitial space were visible. LA-I shows a panoramic view of seminiferous tubules demonstrating the co-existence of largely normal and damage tubules with a different degree of affectation. Tubular limits were completely restored. In many cases it is possible to see normal germ cells and intra-tubular spermatozoa while in some others an accumulation of germ cells and debris are also visualized. Some hyper-picnotic nucleuses within the luminal spaces are visible, although in a significant lesser extension to that observed for PM-treated animals. LA-II, III shows almost normal histology with presence of germ cells and intra-luminal spermatozoa. The interstitial space is conserved showing Leydig cells, mostly of normal appearance.

Considering that LA and Toc are widely used in humans, a brief discussion of the dosage used is warranted. A study of the toxic effect of LA administration in rats demonstrated that it is biosafe for 2 years at doses up to 180 mg/kg day (Cremer et al., 2006; Gu et al., 2010). For chronic treatment (up to 5 years) in humans, LA is usually indicated in three daily doses of 200 mg each, for patients of approximately 70 kg this is equivalent to 9 mg LA/kg bw (Jacob et al., 1999; López-D'alessandro and Escovich, 2011; Ruessmann, 2009); however, in some clinical circumstances this indication is increased to 1800 mg/day (26 mg LA/kg bw) (Ziegler et al., 2006), 3600 mg/day (51 mg LA/bw) (Ziegler et al., 1995,1999) or even higher (daily doses of 600–800 mg LA administered intravenously) (Evans and Goldfine, 2000; Petersen Shay et al., 2009; Ziegler, 2004). Pharmacological and clinical studies have demonstrated that LA is safe for humans at doses of 1800–2400 mg/day (26–35 mg LA/kg bw) for at least 3 months (Petersen Shay et al., 2009; Yadav et al., 2010), even in patients with severe renal failure (Teichert et al., 2005). Furthermore, it has been demonstrated that the rapid gastrointestinal uptake of LA and its appearance in plasma is followed by an equally rapid clearance (Petersen Shay et al., 2009). After the i.p. administration of 100 mg LA/kg bw, the antioxidant is primarily accumulated in liver, heart and skeletal muscle and it efficiently crosses the blood–brain barrier (Arivazhagan et al., 2002; Chng et al., 2009).

The toxicity of α-Toc has largely been studied in humans and remains a matter of debate (Finley et al., 2011). The most widely accepted theory is that Toc has a high biosafety level because the UL (Tolerable Upper Intake Level) for vitamin E in humans is 1000 mg daily. Thus, following the opinion of the Scientific Committee on Food of the European Commission for Health and Consumer Protection (SCF, 2003), the clinical use of this antioxidant appears to have no adverse effects even at doses as high as 1000 mg daily, with the sole exception of a probable increase in bleeding time due to the already described inhibition of prostacyclin biosynthesis (Halliwell and Gutteridge, 1999).

In the present work, in addition to the pesticide-induced oxidative damage to proteins (PCs) and lipids (TBARS), we also observed a higher concentration of [NO*x*] as the end-metabolic product directly related to peroxinitrite overproduction. The observed increases in the levels of [NO*x*] could be the consequence of iNOS activation, which is induced especially under high OS conditions (Halliwell and Gutteridge, 1999). This phenomenon adds a new dimension to the damage associated with pesticides since it indicates the establishment of nitrosative stress derived from the reaction between NO and superoxide, giving rise to the powerful pro-oxidant peroxinitrite (Halliwell and Gutteridge, 1999). Interestingly, a study by Feng Liang and Akaike (2000) demonstrated that LA could be a potent inhibitor of iNOS, even more effective than N^G-monomethyl-L-arginine, without any

demonstrable cytotoxicity. Our results on the effect of LA on [NO*x*] levels are in agreement with those of Freitas (2009). However, to the best of our knowledge, there is no evidence that Toc might act as an inhibitor of iNOS activation. The protective role of Toc (if any) against nitrate+nitrite toxicity is likely to be indirect, through the decrease in superoxide availability, especially in combination with selenium (Chow and Hong, 2002).

Concerning the mechanism(s) involved in agrochemical-induced damage to male fertility, some authors have argued that following repeated exposures changes induced by residual pesticides – in germ cell morphology and function – might result in male infertility (Aitken, 2006) mostly though a hormonal-disruptor mechanism of action (Sarkar et al., 2000). It was previously reported that D 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., 2000; Afifi et al., 1991; Hurtado de Catalfo et al., 2011; Astiz et al., 2009a,b, 2012) and mice (Farag et al., 2007). Moreover, a previous work from our lab demonstrates that, D 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 PGF2a) and also the inhibition of steroidogenic enzymes 17βHSD and 3βHSD (Astiz et al., 2009a,b, 2012). Recent studies in rats suggest that, the exposure to G 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, G 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 G had difficulty to conceive and also showed a higher rate of miscarriage (Arbuckle et al., 2001; Caglar and Kolankaya, 2008a,b). In addition, zineb produces a decrease in mouse fertility performance due to alterations in male and female pronuclei formation (Rossi et al., 2006). However, despite these evidences, the exact mechanism of agrochemical-induced gonadal dysfunction remains to be completely elucidated.

Inflammation might play a key role in the damages observed. Even though the antioxidant properties of LA are widely recognized, opinions differ as to the implications of this. A limited number of studies on the pro-oxidant action of both LA and its metabolite, dihydrolipoic acid (DHLA), have been carried out in recent years (Cakatay, 2006; Moini et al., 2002; Petersen Shay et al., 2009), sparking controversy about the beneficial effect of this antioxidant. In our experimental model the antioxidant protection exerted by LA was evident at both the systemic (peripheral plasma) and local (testis homogenates) levels. This finding is in agreement with the great bulk of evidence proposing lipoate as the most powerful and versatile antioxidant. For this reason LA has been labeled "the antioxidant of antioxidants" (Bilska and Włodek, 2005). Scientific literature abounds with examples of the multiple protective actions displayed by LA, including its activity as a metal chelator agent and ROS/RNS scavenger, and its role in the preservation of other antioxidant molecules from the water- and soluble-cell compartment (Bilska and Włodek, 2005; Holmquist et al., 2007). Moreover, the dose-dependent protective effect of LA – as evidenced by the reduction in the levels of the damage biomarkers – was even observed in control animals not exposed to the pro-oxidative condition produced by PM administration. This fact *per se* is of interest from the clinical point of view, suggesting that LA could be considered as a supplement to be used in cases of suspected sub-clinical free radical formation, for example exposure to environmental pollutants that cause OS (Arnal et al., 2011; Astiz et al., 2011; Cristalli et al., 2011).

Similarly, experimental evidence suggests that excessive amounts of Toc might produce pro-oxidative events (Finley et al., 2011) mostly due to a consumption of ascorbate which is essential for the regeneration of the α-tocopheryl radical. However, as happens with LA, in our experimental conditions Toc displayed antioxidant properties in coincidence with the largely accepted action of this vitamin (SCF, 2003). Also, it is well known that α-Toc can be indirectly recycled by LA through the reduction of dihydroascorbate to ascorbate (Guo and Packer, 2000; Kagan et al., 1992). We observed a restoration of the Toc concentration by LA in the present study; this fact may acquire additional importance in the testis, where PUFAs play a critical and relevant role from a number of physiological points of view (Astiz et al., 2009, 2011; Hurtado de Catalfo and Alaniz, 2008; Hurtado de Catalfo et al., 2011). Moreover, LA prevents the reoxidation of GSH and protects glutathione-related enzymes from peroxidative or nitrosative damage (Arivazhagan et al., 2000).

As stated before, Toc has proven antioxidant properties as reflected in the increment observed in FRAP assay values. Although some experimental research has suggested that Toc might have anti-inflammatory action (Ekstrand-Hammarström et al., 2007; Vázquez et al., 2012), we found no significant effect on prostaglandin production or hydroxysteroid dehydrogenase enzyme activities. On the contrary, administration of LA produced a strong decrease in the production and/or in the lower catabolic rate of PGE_2 and $PGF_{2\alpha}$ (or both), resulting in a clear anti-inflammatory condition reflected at systemic level. This finding is in agreement with the previously reported effect of LA as an inhibitor of inflammation in many experimental systems (Cho et al., 2004; Choi et al., 2010; Deiuliis et al., 2011; Foo et al., 2011; Khabbazi et al., 2012; Kolgazi et al., 2007; Odabasoglu et al., 2011). The anti-inflammatory effect of LA likely resides in the fact that COX-2 and phospholipase A_2 – the main source of the substrate for prostaglandin biosynthesis – are both inhibited by LA (Chaudhary et al., 2011; Ha et al., 2006; Ho et al., 2007; Jameel et al., 2006). Pesticide-associated inflammation might be caused indirectly through the excessive formation of reactive species derived from oxygen (ROS) and/or nitrogen (RNS). Thus, administration of LA could have important implications in clinical situations hallmarked by pesticide (or pollutant)-induced inflammation of the male reproductive system. We have previously demonstrated that testosterone biosynthesis depends at least on the effect exerted by D on the biological activity of the mitochondrial StAR protein (Astiz et al., 2009a,b). StAR activity is indirectly dependent on the level of PGE_2 and $PGF_{2\alpha}$, both derived from arachidonate through the intervention of the phospholipase A_2. Thus, the administration of a COX-2 inhibitor such as Rofecoxib® produces both normalization of prostaglandin levels and strong reversion of the D-induced inhibition of StAR biological activity (Astiz et al., 2009a,b). These findings are also in agreement with our present results on the anti-inflammatory effect displayed by LA.

Increased OS in response to PM exposure is not only involved in pro-inflammatory conditions but also in the activation of the programmed cell death pathway with the subsequent modification of the cell cycle phases (Chandra et al., 2000). The experimental evidence demonstrating that pesticides are able to modify the histological structure of the testis is in agreement with our results. The considerable damage to the germ cells correlated with the decreased production in spermatozoa (in count and with altered morphology) previously demonstrated in our previous paper (Hurtado de Catalfo et al., 2011) and the damage to the interstitial cell population is also in agreement with the

diminished testosterone biosynthesis. Both abnormalities were efficiently prevented by co-administration with LA. Previous papers demonstrating the beneficial effect of LA administration in testis from rats receiving a mixture of the pesticides chlorpyrifos and fenitrothion (Gawish, 2010) or the administration of 4-tert-octylphenol (Othman et al., 2012) reinforces the promising use of this antioxidant as a tool to prevent the histological damage induced by diverse pesticides. Since human life without pesticide exposure is practically inconceivable, the establishment of lower MRLs (Maximum Residues Levels) in food together with antioxidant supplementation of diet (especially those of an amphiphilic nature such as lipoate) remains a valid preventive strategy (González-Pérez and González-Castaneda, 2006).

5. Conclusion

The present results show that LA is more efficient than Toc in protecting rat testis against the deleterious effects of pesticide exposure suggesting that pesticides act through other mechanism(s) of action than generating OS. LA has excellent antioxidant properties in both lipophilic and hydrophilic testis cell environments due to its ability to prevent oxidative damage to lipids and proteins; it also appears to block the pro-inflammatory action of pesticides and is able to efficiently restore the histological condition of the testis. Our experimental protocol constitutes a promising model for investigating questions relating to the dosage and administration routes, biomarkers of damage with probable clinical utility, and the impact of LA administration on the components of the antioxidant defense system in testis.

Acknowledgments

This study was supported by a grant [PIP #0697] from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. We would like to thank Vet. J. M. Lofeudo for animal handling, clinical evaluation, and collaboration with brain dissections, and Mrs. C. Zardis de Cobeñas, E. Illara de Bozzolo, C. Pallanza de Stringa, and N. Cristalli for their excellent technical assistance.

References

Afifi, N.A., Ramadan, A., el-Aziz, M.I., Saki, E.E., 1991. Influence of dimethoate on testicular and epididymal organs, testosterone plasma level and their tissue residues in rats. Dtsch. Tierarztl. Woch. 98, 419–423.

Aitken, R.J., 2006. Sperm functional tests and fertility. Int. J. Andrology 29, 6975.

Aitken, R.J., Barker, M.A., 2006. Oxidative stress, sperm survival and fertility control. Mol. Cell. Endocrinol. 250, 66–69.

Arbuckle, T.E., Lin, Z., Mery, L.S., 2001. An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. Environ. Health Perspect. 109, 851–857.

Arivazhagan, P., Juliet, P., Pannerselvam, C., 2000. Effect of DL-α-lipoic acid on the status of lipid peroxidation and antioxidants in aged rats. Pharmacol. Res. 41, 299–303.

Arivazhagan, P., Shila, S., Kumaran, S., Panneerselvam, C., 2002. Effect of DL-α-lipoic acid on the status of lipid peroxidation and antioxidant enzymes in various brain regions in aged rats. Exp. Gerontol. 37, 803–811.

Arnal, N., Astiz, M., Alaniz, M.J.T., de, Marra, C.A., 2011. Clinical parameters and biomarkers of oxidative stress in agricultural workers who applied copper-based pesticides. Ecotoxicol. Environ. Saf. 74, 1779–1786.

Astiz, M., Hurtado de Catalfo, G.E., Alaniz, M.J.T., de, Marra, C.A., 2012. Exogenous arachidonate restores the dimethoate-induced inhibition of steroidogenesis in rat interstitial cells. Lipids 47, 557–569.

Astiz, M., Hurtado de Catalfo, G.E., Alaniz, M.J.T., de, Marra, C.A., 2009a. Involvement of lipids in dimethoate-induced inhibition of testosterona biosynthesis in rat interstitial cells. Lipids 44, 703–718.

Astiz, M., Alaniz, M.J.T., de, Marra, C.A., 2009b. The impact of simultaneous intoxication with agrochemicals on the antioxidant defense system in rat. Pestic. Biochem. Physiol. 94, 93–99.

Astiz, M., Alaniz, M.J.T., de Marra, C.A., 2009c. Effect of pesticides on cell survival in rat liver and brain. Ecotoxicol. Environ. Safety 72, 2025–2032.

Astiz, M., Alaniz, M.J.T., de Marra, C.A., 2009d. Antioxidant defense system in rats simultaneously intoxicated with agrochemicals. Environ. Toxicol. Pharmacol. 28, 465–473.

Astiz, M., Anal, N., Alaniz, M.J.T., de, Marra, C.A., 2011. Occupational exposure characterization in professional sprayers: clinical utility of oxidative stress biomarkers. Environ. Toxicol. Pharmacol. 32, 249–528.

Bagchi, D., Bagchi, M., Hassoun, E.A., Stohs, S.J., 1995. In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104, 129–140.

Bagnati, M., Bordone, R., Perugini, C., Cau, C., Albano, E., Bellomo, G., 1998. Cu(I) availability paradoxically antagonizes antioxidant consumption and lipid peroxidation during the initiation phase of copper-induced LDL oxidation. Biochem. Biophys. Res. Commun. 253, 235–240.

Banerjee, B.D., Seth, A., Bhattacharya, A., Pasha, S.T., Chakraborty, A.K., 1999. Biochemical effects of some pesticides on lipid peroxidation and free radical scavengers. Toxicol. Lett. 107, 33–47.

Barlow, B.K., Lee, D.W., Cory-Slechta, D.A., Opanashuk, L.A., 2005. Modulation of antioxidant defence systems by the environmental pesticide Maneb in dopaminergic cells. Neurotoxicology 26, 63–75.

Basrur, P.K., 2006. Disrupted sex differentiation and feminization of man and domestic animals. Environ. Res. 100, 18–38.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal. Biochem. 239, 70–76.

Beuret, C.J., Zirulnik, F., Gimenez, M.S., 2005. Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. Reprod. Toxicol. 19, 501–504.

Bilska, A., Włodek, L., 2005. Lipoic acid—the drug of the future? Pharmacol Rep. 57, 570–577.

Bolognesi, C., Morasso, G., 2000. Genotoxicity of pesticides: potential risk for consumers. Trends Food Sci. Tech. 11, 182–187.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buttriss, J.L., Diplock, A.T., 1984. High-performance liquid chromatography methods for vitamin E in tissues. Methods Enzymol. 105, 131–138.

Caglar, S., Kolankaya, D., 2008a. The effect of sub-acute and sub-chronic exposure of rats to the glyphosate-based herbicide Roundup. Environ. Toxicol. Pharmacol. 25, 57–62.

Caglar, S., Kolankaya, D., 2008b. The effect of sub-acute and sub-chronic exposure of rats to the glyphosate-based herbicide Roundup. Environ. Toxicol. Pharmacol. 25, 57–62.

Cakatay, U., 2006. Pro-oxidant actions of alpha-lipoic acid and dihydrolipoic acid. Med. Hypotheses 66, 110–117.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29, 323–333.

Chaudhary, P., Marracci, G., Yu, X., Galipeau, D., 2011. Morris B, Bourdette D. Lipoic acid decreases inflammation and confers neuroprotection in experimental autoimmune optic neuritis. J. Neuroimmunol. 233, 90–96.

Chng, H.T., New, L.S., Neo, A.H., Goh, C.W., Browne, E.R., Chan, E.C.Y., 2009. Distribution study of orally administered lipoic acid in rat brain tissues. Brain Res. 1251, 80–86.

Cho, Y.S., Lee, J., Lee, T.H., Lee, E.Y., Lee, K.U., Park, J.Y., Moon, H.B., 2004. Alpha-Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. J. Allergy Clin. Immunol. 114, 429–435.

Choi, Y.H., Chai, O.H., Han, E.H., Choi, S.Y., Kim, H.T., Song, C.H., 2010. Lipoic acid suppresses compound 48/80-induced anaphylaxis-like reaction. Anat. Cell. Biol. 43, 317–324.

Chow, C.K., Hong, C.B., 2002. Dietary vitamin E and selenium and toxicity of nitrate and nitrite. Toxicology 180, 195–207.

Cory-Slechta, D.A., 2005. Studying toxicants as single chemicals: Does this strategy adequately identify neurotoxic risk? Neurotoxicology 26, 491–510.

Cremer, D.R., Rabeler, R., Roberts, A., Lynch, B., 2006. Long-term safety of α-lipoic acid (ALA) consumption: a 2-year study. Regul. Toxicol. Pharmacol. 46, 193–201.

Cristalli, D.O., Arnal, N., Marra, F.A., Tacconi de Alaniz, M.J., Marra, C.A., 2011. Peripheral markers in neurodegenerative patients and their first-degree relatives. J. Neurol. Sci. 414, 48–56.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R., Milzani, A., 2006. Biomarkers of oxidative damage in human disease. Clin. Chem. 52, 601–623.

Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J., Dalsenter, P.R., Langeloh, A., 2007. Pre and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 681, 665–673.

Daruich, J., Zirulnik, F., Gimenez, M.S., 2001. Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. Environ. Res. 85, 226–231.

Deiuliis, J.A., Kampfrath, T., Ying, Z., Maiseyeu, A., Rajagopalan, S., 2011. Lipoic acid attenuates innate immune infiltration and activation in the visceral adipose tissue of obese insulin resistant mice. Lipids 46, 1021–1032.

Di Monte, D., 2003. The environment and Parkinson's disease: is the nigrostriatal system preferentially targeted by neurotoxins? Lancet Neurol. 2, 531–537.

Ekstrand-Hammarström, B., Österlund, C., Lilljeöök, B., Bucht, A., 2007. Vitamin E down-regulates mitogen-activated protein kinases, nuclear factor kB and inflammatory responses in lung epithelial cells. Clin. Exp. Immunol. 147, 359–369.

Evans, J.L., Goldfine, I.D., 2000. Alpha-lipoic acid: a multifunctional antioxidant that improves insulin sensitivity in patients with type 2 diabetes. Diabetes Technol. Ther. 2, 401–413.

Farag, A.T., El-Aswad, A.F., Shaaban, N.A., 2007. Assessment of reproductive toxicity of orally administered technical dimethoate in male mice. Reprod. Toxicol. 23, 232–238.
Feng Liang, J., Akaike, T., 2000. Inhibition of nitric oxide synthesis in primary cultured mouse hepatocytes by α-lipoic acid. Chem. Biol. Interact. 124, 53–60.
Finley, J.W., Kong, A.N., Hintze, K.J., Jeffery, E.H., Ji, L.L., Lei, X.G., 2011. Antioxidants in foods: state of the science important to the food industry. J. Agr. Food Chem. 59, 6837–6846.
Fitsanakis, V.A., Amarnath, V., Moore, J.T., Montine, K.S., Zhang, J., Montine, T.J., 2002. Catalysis of catechol oxidation by metal-dithiocarbamate complexes in pesticides. Free Radic. Biol. Med. 33, 1714–1723.
Foo, N.P., Lin, S.H., Lee, Y.H., Wu, M.J., Wang, Y.J., 2011. α-Lipoic acid inhibits liver fibrosis through the attenuation of ROS-triggered signalling in hepatic stellate cells activated by PDGF and TGF-β. Toxicology 282, 39–46.
Freitas, R.M., 2009. The evaluation of effects of lipoic acid on the lipid peroxidation, nitrite formation and antioxidant enzymes in the hippocampus of rats after pilocarpine-induced seizures. Neurosci. Lett. 455, 140–144.
Gawish, A.M., 2010. The protective role of alpha-lipoic acid against pesticides induced testicular toxicity. Histopathological and histochemical studies. Life Sci. J. 7, 117–124.
González-Pérez, O., González-Castaneda, R.E., 2006. Therapeutic perspectives on the combination of α-lipoic acid and vitamin. E. Nutr. Res. 26, 1–5.
Gu, X.M., Zhang, S.S., Wu, J.C., Tang, Z.Y., Lu, Z.Q., Li, H., Liu, C., Chen, L., Ning, G., 2010. Efficacy and safety of high-dose α-lipoic acid in the treatment of diabetic polyneuropathy. Zhonghua Yi Xue Za Zhi 21, 2473–2476.
Guo, Q., Packer, L., 2000. Ascorbate-dependent recycling of the vitamin E homologue trolox by dihydrolipoate and glutathione in murine skin homogenates. Free Radic. Biol. Med. 29, 368–374.
Ha, H., Lee, J.H., Kim, H.N., Kim, H.M., Kwak, H.B., Lee, S., Kim, H.H., Lee, Z.H., 2006. alpha-Lipoic acid inhibits inflammatory bone resorption by suppressing prostaglandin E2 synthesis. J. Immunol. 176, 111–117.
Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, Third Edition Oxford Science Publications.
Heikkila, R.E., Cabbat, F.S., Cohen, G., 1976. In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. J. Biol. Chem. 251, 2182–2185.
Ho, Y.S., Lai, C.S., Liu, H.I., Ho, S.Y., Tai, C., Pan, M.H., Wang, Y.J., 2007. Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation. Biochem. Pharmacol. 73, 1786–1795.
Holmquist, L., Stuchbury, G., Berbaum, K., Muscat, S., Young, S., Hager, K., Engel, J., Münch, G., 2007. Lipoic acid as a novel treatment for Alzheimer's disease and related dementias. Pharmacol. Ther. 113, 154–164.
Hurtado de Catalfo, G., Alaniz, M.J.T., de, Marra, C.A., 2008. Dietary lipids modify the redox homeostasis and the steroidogenic status in rat testis. Nutrition 24, 717–726.
Hurtado de Catalfo, G.E., Astiz, M., Alaniz, M.J.T., de, Marra, C.A., 2011. Frequently-used agrochemicals lead to functional and morphological alterations in spermatozoa in rats. J. Toxicol. Environ. Health Sci. 3, 180–192.
Jacob, S., Ruus, P., Hermann, R., Tritschler, H.J., Maerker, E., Renn, W., Augustin, H.J., Dietze, G.J., Rett, K., 1999. Oral administration of RAC-alpha-lipoic acid modulates insulin sensitivity in patients with type-2 diabetes mellitus: a placebo-controlled pilot trial. Free Radic. Biol. Med. 27, 309–314.
Jameel, M.L., Shekhar, M.A., Vishwanath, B.S., 2006. α-lipoic acid: an inhibitor of secretory phospholipase A_2 with anti-inflammatory activity. Life Sci. 80, 146–153.
John, S., Kale, M., Rathore, N., Bhatnagar, D., 2001. Protective effect of vitamin E in dimethoate and malathion induced oxidative stress in rat erythrocytes. J. Nutr. Biochem. 12, 500–504.
Kagan, V.E., Serbinova, E.A., Forte, T., Scita, G., Packer, L., 1992. Recycling of vitamin E in human low density lipoproteins. J. Lipid Res. 33, 385–397.
Kesavachandran, C.N., Fareed, M., Pathak, M.K., Bihari, V., Mathur, N., Srivastava, A.K., 2009. Adverse health effects of pesticides in agrarian populations of developing countries. Rev. Environ. Contam. Toxicol. 200, 33–52.
Khabbazi, T., Mahdavi, R., Safa, J., Pour-Abdollahi, P., 2012. Effects of alpha-lipoic acid supplementation on inflammation, oxidative stress, and serum lipid profile levels in patients with end-stage renal disease on hemodialysis. J. Ren. Nutr. 22, 244–250.
Kim, M.S., Park, J.Y., Namkoong, C., Jang, P.G., Ryu, J.W., Song, H.S., Yun, J.Y., Namgoong, I.S., Ha, J., Park, I.S., Lee, I.K., Viollet, B., Youn, J.H., Lee, H.K., Lee, K.U., 2004. Anti-obesity effect of α-lipoic acid mediated by suppression of hypothalamic AMP activated protein kinase. Nat. Med 10, 727–733.
Kolgazi, M., Jahovic, N., Yüksel, M., Ercan, F., Alican, I., 2007. Alpha-lipoic acid modulates gut inflammation induced by trinitrobenzene sulfonic acid in rats. J. Gastroenterol. Hepatol. 22, 1859–1865.
Liu, B., Gao, H.-M., Hong, J.S., 2003. Parkinson's disease and exposures to infectious agents and pesticides and the occurrence of brain injuries: role of neuroinflammation. Environ. Health Persp. 111, 1065–1073.
López-D'alessandro, E., Escovich, L., 2011. Combination of alpha lipoic acid and gabapentin, its efficacy in the treatment of burning mouth syndrome: a randomized, double-blind, placebo controlled trial. Med. Oral Patol. Oral Cir. Bucal. 16, e635–e640.
Maczurek, A., Hager, K., Kenklies, M., Sharman, M., Martins, R., Engel, J., Carlson, D.A., Münch, G., 2008. Lipoic acid as an anti-inflammatory and neuroprotective treatment for Alzheimer s disease. Adv. Drug Deliver. Rev. 60, 1463–1470.
Marangon, K., Devaraj, S., Tiroshi, O., Packer, L., Jialal, I., 1999. Comparison of the effect of α-Lipoic acid and α-tocopherol supplementation on measures of oxidative stress. Free Radic. Biol. Med. 27, 1114–1121.
Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5, 62–71.
Moini, H., Packer, L., Saris, N.E., 2002. Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. Toxicol. Appl. Pharmacol. 182, 84–90.
Moser, V.C., Simmons, J.E., Gennings, C., 2006. Neurotoxicological interactions of five-pesticide mixture in prewealing rats. Toxicol. Sci. 92, 235–245.
Murugesan, P., Kanagaraj, P., Yuvaraj, S., Balasubramanian, K., Aruldhas, M.M., Arunakaran, J., 2005. The inhibitory effects of polychlorinated biphenyl Aroclor 1254 on Leydig cell LH receptors, steroidogenic enzymes and antioxidant enzymes in adult rats. Reprod Toxicol. 20, 117–126.
Nagamatsu, M., Nickander, K.K., Schmelzer, J.D., Raya, A., Wittrock, D.A., Tritschler, H., Low, P.A., 1995. Lipoic acid improves nerve blood flow, reduces oxidative stress, and improves distal nerve conduction in experimental diabetic neuropathy. Diabetes Care 18, 1160–1167.
National Research Council. Guide for the care and use of laboratory animals. 1985. Publication No 85-23 (rev.). National Institute of Health, Bethesda, MD.
Odabasoglu, F., Halici, Z., Aygun, H., Halici, M., Atalay, F., Cakir, A., Cadirci, E., Bayir, Y., Suleyman, H., 2011. α-Lipoic acid has anti-inflammatory and anti-oxidative properties: an experimental study in rats with carrageenan-induced acute and cotton pellet-induced chronic inflammations. Br. J. Nutr. 105, 31–43.
Othman, A.I., El-Missiry, M.A., Koriem, K.M., El-Sayed, A.A., 2012. Alfa-lipoic acid protects testosterone secretion pathway and sperm quality against 4-tert-octylphenol induced reproductive toxicity. Ecotoxicol. Environ. Saf. 81, 76–83.
Petersen Shay, K., Moreau, R.F., Smith, E.J., Smith, A.R., Hagen, T.M., 2009. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. Biochim. Biophys. Acta 1790, 1149–1160.
Reznick, A.D., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.
Rossi, G., Buccione, R., Baldassarre, M., Macchiarelli, G., Palmerini, M.G., Cecconi, S., 2006. Mancozeb exposure in vivo impairs Mouse oocyte fertilizability. Reprod. Toxicol. 21, 216–219.
Ruessmann, H.J., 2009. Switching from pathogenetic treatment with alpha-lipoic acid to gabapentin and other analgesics in painful diabetic neuropathy: a real-world study in outpatients. German Society of out patient diabetes centres AND (Arbeitsgemeinschaft niedergelassener diabetologisch tätiger Arzte e.V.). J. Diabetes Complicat. 23, 174–177.
Salem, M.H., Abo-Elezz, Z., Abd-Allah, G.A., Hassan, G.A., Shaker, N., 1988. Effect of organophosphorous (dimethoate) and pyretroid (deltamethrin) pesticides on semen characteristics in rabbits. J. Envirom. Sci. Health B 23, 279–290.
Sarkar, R., Mohanakumar, K.P., Chowdhury, M., 2000. Effects of an organophosphate pesticide, quinalphos, on the hypothalamo-pituitary-gonadal axis in adult male rats. J. Reprod. Fertil. 118, 29–38.
SCF, Scientific Committee on Food, 2003. European Commission. Health & Consumer Protection (Directorate-General), Brussels – Belgium.
Sharma, Y., Bashir, S., Irshad, M., Nag, T.C., Dogra, T.D., 2005a. Dimethoate-induced effects on antioxidant status of liver and brain of rats following sub chronic exposure. Toxicology 215, 173–181.
Sharma, Y., Bashir, S., Irshad, M., Datta Gupta, S., Dogra, T.D., 2005b. Effects of acute dimethoate administration on antioxidant status of liver and brain of experimental rats. Toxicology 206, 49–57.
Sheweita, S.A., Tilmisany, A.M., Al-Sawaf, H., 2005. Mechanism of male infertility. Curr. Drug. Metab. 6, 495–501.
Sikka, S.C., 2001. Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem. 8, 851–862.
Sinclair, S., 2000. Male infertility: nutritional and environmental considerations. Altern. Med. Rev. 5, 28–38.
Tang, J., Wingerchuk, D.M., Crum, B.A., Rubin, D.I., Demaerschalk, B.M., 2007. Alpha-Lipoic acid may improve symptomatic diabetic polyneuropathy. Neurologist 13, 164–167.
Teichert, J., Tuemmers, T., Achenbach, H., Preiss, C., Hermann, R., Ruus, P., Preiss, R., 2005. Pharmacokinetics of alpha-lipoic acid in subjects with severe kidney damage and end-stage renal disease. J. Clin. Pharmacol. 45, 313–328.
Urdaneta de Romero, A., Madrid-Bury, N., Rodríguez-Márquez, J., Aranguren-Méndez, J., Camacho Bracho, J., González-Stagnaro, C., Castejón-Sandoval, C., 1998. Histopathological and morphometry of testis in 5/8 Holstein and 5/8 Brown Swiss crossbread young bulls. Rev. Cient. FCV-LUZ 2, 163–176.
Vázquez MC, Balboa E, Alvarez AR, Zanlungo S. 2012. Oxidative stress: a pathogenic mechanism for Niemann-Pick type C disease. Oxid Med Cell Longev. 2012: article ID 205713(11 pages).
Walsh, L.P., Webster, D.R., Stocco, D.M., 2000. Dimethoate inhibits esteroidogenesis by disrupting transcription of the esteroidogenic acute regulatory (StAR) gene. J. Endocrinol. 167, 253–263.
Yadav, V., Marracci, G.H., Munar, M.Y., Cherala, G., Stuber, L.E., Alvarez, L., Shinto, L., Koop, D.R., Bourdette, D.N., 2010. Pharmacokinetic study of lipoic acid in multiple sclerosis: comparing mice and human pharmacokinetic parameters. Mult. Scler. 16, 387–397.
Yagi, K., 1976. A simple flourometric assay for lipoperoxide in blood plasma. Biochem. Med. 15, 212–216.
Ziegler, D., 2004. Thioctic acid for patients with symptomatic diabetic polyneuropathy: a critical review. Treat. Endocrinol 3, 173–189.
Ziegler, D., Ametov, A., Barinov, A., Dyck, P.J., Gurieva, I., Low, P.A., Munzel, U., Yakhno, N., Raz, I., Novosadova, M., Maus, J., Samigullin, R., 2006. Oral treatment with alpha-lipoic acid improves symptomatic diabetic polyneuropathy: the SYDNEY 2 trial. Diabetes Care 29, 2365–2370.

Ziegler, D., Hanefeld, M., Ruhnau, K.J., Meissner, H.P., Lobisch, M., Schütte, K., Gries, F.A., 1995. Treatment of symptomatic diabetic peripheral neuropathy with the anti-oxidant alpha-lipoic acid. A 3-week multicentre randomized controlled trial (ALADIN Study). Diabetologia 38, 1425–1433.

Ziegler, D., Reljanovic, M., Mehnert, H., Gries, F.A., 1999. Alpha-lipoic acid in the treatment of diabetic polyneuropathy in Germany: current evidence from clinical trials. Exp. Clin. Endocrinol. Diabetes. 107, 421–430.

Zini, A., Kamal, K., Phang, D., Willis, J., Jarvi, K., 2001a. Biologic variability of sperm DNA denaturation in infertile men. Urology 58, 258–261.

Zini, A., Kamal, K.M., Phang, D., 2001b. Free thiols in human spermatozoa: correlation with sperm DNA integrity. Urology 58, 80–84.