

## Involvement of Lipids in Dimethoate-Induced Inhibition of Testosterone Biosynthesis in Rat Interstitial Cells

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Received: 25 November 2008 / Accepted: 13 May 2009 / Published online: 5 July 2009  
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**Abstract** The mechanism involved in the inhibition of testosterone (Te) biosynthesis after a sub-chronic exposure to low doses of dimethoate (D) was studied in rat interstitial cells (IC). Expression of COX-2 in IC isolated from D-treated rats increased by 44% over C data, while transcription of StAR decreased by approx. 50% and the expression of this protein was diminished by approximately 40%. PGE<sub>2</sub> and PGF<sub>2α</sub> were increased by 61 and 78%, respectively. Te concentration decreased by 49% in IC homogenates. Concomitantly, plasma concentration of LH and FSH both increased. Arachidonic acid (ARA) and C<sub>22</sub> fatty acyl chains in phospholipids from IC mitochondrial fraction decreased by approx. 30% after D treatment. Protein carbonyls, lipoperoxides and nitrite content increased while α-tocopherol and the antioxidant capacity of the soluble cellular fraction decreased significantly. Stimulation with h-CG 10 nM overnight failed to overcome the inhibition caused by D on both Te biosynthesis and 3β- and 17β-hydroxysteroid dehydrogenases. Decreased Te biosynthesis may be attributed to (1) inhibition of StAR protein activity due to the stimulation of COX-2 and the overproduction of PGF<sub>2α</sub>, (2) decreased stimulatory effect of ARA on StAR with a subsequent reduction in the availability of CHO for the androgenic pathway, and/or (3) indirect inhibition of steroidogenic enzymes by a lower transcriptional rate caused by elevated

PGF<sub>2α</sub>. Rofecoxib administration prevents the deleterious effect(s) exerted by D.

**Keywords** Arachidonic acid · COX-2 · Dimethoate · Oxidative stress · Prostaglandins · Rat interstitial cells · StAR · h-CG · Rofecoxib · TROLOX

### Abbreviations

C	Control group
CM	Cytoplasmic membrane
D	Dimethoate
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
LPO	Lipid peroxidation
MDA	Malonedialdehyde
N	Nucleus
OS	Oxidative stress
RNS	Reactive nitrogen species
PS	Protein synthesis
R	Rofecoxib
ROS	Reactive oxygenated species
StAR	Steroidogenic acute regulatory protein
T	TROLOX®
TBARS	Thiobarbituric acid-reactive substances
Te	Testosterone

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### Introduction

The growing amount of experimental evidence documenting endocrine dysfunction in male wildlife has led to the hypothesis that environmental contaminants adversely affect testosterone production and caused deleterious

effects on the male reproductive system [1]. In an interesting work, Carlsen et al. [2] reported that a significant worldwide decline in semen quality has occurred over the past 50 years. Retrospective studies performed in sperm banks from Paris [3] and London [4] have supported this conclusion. The hypothesis of environmental pollution as a causative factor is casually related to higher declines in sperm quality observed in developed countries characterized by important agrotechnical activities [5, 6]. Other authors reported that environmental chemicals can mimic estrogen action and also disrupt androgen signaling pathways [6]. Moreover, alterations were also observed not only in reproductive performance, but also in the incidence of cryptorchidism and testicular cancer [7]. Many contaminants demonstrated antiandrogenic activity both in whole animals and in receptor binding-in vitro assays [1]. Important evidence for this mechanism of action was obtained with the herbicide Linuron. This chemical agent binds to the androgen receptor with an affinity similar to that of flutamide, which is a potent antiandrogenic drug. As a result, feedback control to the pituitary is interrupted and serum LH and estrogen concentrations increase significantly. The ultimate outcome of Linuron exposure is Leydig cell cancer due to over-stimulation of steroidogenesis [8]. Several pesticides and their metabolites have similar effects [6, 9]. Currently, the levels of environmental exposure to these substances and their derivatives are completely unknown. Thus, predictions about alterations in male reproductive function or fertility cannot be made [6, 10]. This fact has generated considerable public and scientific concern about the relationship existing between environmental contaminants and alterations to the male reproductive system [11].

Some of the most used agrochemicals have been shown to disturb pro-oxidant/anti-oxidant balance in testicular cells and consequently decrease the androgen production [6]. Free radical generation in response to toxicants [12] plays different and complex roles in the Leydig cell physiology [6]. For example, high levels of corticosterone produced during oxidative stress may bring about, in part, the decline in testosterone biosynthesis since this steroid is able to induce Leydig cell apoptosis [13]. In addition, testosterone production is profoundly affected by the prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) level. The inducible enzyme cyclooxygenase-2 (COX-2) is responsible for the biosynthesis of  $PGF_{2\alpha}$  from its precursor arachidonic acid (ARA).  $PGF_{2\alpha}$  not only inhibits progesterone production but also activates its own biosynthetic pathway by induction of COX-2 via protein kinase C in an autoamplification cascade [14]. In this scenario it is possible to assume that pesticides induce oxidative stress (OS) and disturb the conversion of ARA into  $PGF_{2\alpha}$  via COX-2. In addition, other laboratories have demonstrated that COX-2 and

$PGF_{2\alpha}$  are both inhibitors of Leydig cell steroidogenesis [14, 15].

In Leydig cells, cholesterol is converted to testosterone through various steps catalyzed by different enzymes [16]. The delivery of free cholesterol to the inner mitochondrial membrane is the true rate-limiting step of steroidogenesis and it is mediated mainly by the steroidogenic acute-regulatory protein (StAR) [16, 17]. The biological activity of this protein depends on many factors such as ARA,  $PGF$  levels, COX-2 activity, and free radical production in a complex interrelationship not fully understood yet [14, 15, 18–20]. StAR is also sensitive to various pesticides as demonstrated by Walsh et al. [21].

Some organophosphorus compounds are known to impair fertility, suppress libido, cause testicular degeneration, and a deterioration in semen quality [22]. Dimethoate (D; 2-dimethoxyphosphonylthio-*N*-methylacetamine) is an organophosphorus pesticide extensively used as a systemic insecticide and acaricide which proved to be neurotoxic [22] and to disturb StAR gene expression [21]. Toxicity of D on testicular Leydig cell function is still unexplored. Therefore, in the present study we aimed to investigate the action of a sub-chronic exposure of Wistar rats to low doses of D on Leydig cell steroidogenesis, free radical production, and StAR and COX-2 expression. The testis is the major site for testosterone production. It plays a crucial role in the development of secondary sexual characteristics and initiation, as well as regulation of spermatogenesis. Thus, reduction in testosterone production due to exposure to environmental toxicants (such as D) has the potential to adversely affect normal sexual development in humans.

## Experimental Procedure

### Chemicals

All 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).  $\alpha$ -Tocopheryl-diacetate (TROLOX<sup>®</sup>, 98% pure) and rofecoxib (99% pure) were from Saporitti S.A., Buenos Aires, Argentina. Other chemicals employed were purchased from local commercial sources and they were of analytical grade. Dimethoate (2-dimethoxyphosphonylthio-*N*-methylacetamine) was obtained as a gift from INTA (Castelar, Argentina). Hormone measurements [luteinizing (LH), follicle-stimulating (FSH), total and free testosterone] were performed using commercial kits (KP7CT, KP6CT, KS24CT, and KS33CTN, respectively) from Radim (Radim SpA, Pomezia, Italy).

## Animals and Treatments

Male Wistar rats weighing  $190 \pm 20$  g with specific-pathogen free-certified status were used. Upon arrival, the rats were allowed to acclimatize for a week before starting the experiment. Rats were maintained under controlled conditions of temperature ( $25 \pm 2$  °C), and a normal photoperiod of 12 h dark and 12 h light. They were fed with standard Purina chow from Ganave S.A. (Santa Fe, Argentina) and water ad libitum. Clinical examination together with body weight evaluation was performed every week during the experiment. Animals were randomly divided into two groups of six rats each, assigned as control rats (C) injected i.p. with polyethylene-glycol 400 (PEG-400), or D-treated rats injected with 15 mg D/kg body weight dissolved in PEG-400. All animals were injected three times a week for 5 weeks. The dose used was in accordance with previous reports [23–26]. This model was chosen for simulation of a sub-chronic exposition to low doses of pesticide incorporated from the living environment [27]. Other experiment was designed to study the effect of the antioxidant  $\alpha$ -tocopheryl diacetate (TROLOX<sup>®</sup>) and the COX inhibitor rofecoxib administered alone or in combination with dimethoate. In this experimental protocol TROLOX<sup>®</sup> (T) and rofecoxib (R) were dissolved in PBS with PEG-400 and administered i.p. to groups of 4 rats each at a doses of 12.5 and 5.0 mg/kg body weight, respectively. Two groups of rats received TROLOX<sup>®</sup> (CT) or rofecoxib (CR), other two groups were simultaneously injected with D and T (DT) or D and R (DR). Finally, other groups of animals were injected with the vehicle alone (C), dimethoate alone (D), or a combination of the three drugs (DTR). Animal maintenance and handling were in accordance with the NIH guide for the care and use of laboratory animals [28]. All procedures were approved by the local Laboratory Animal Bioethics Committee, Facultad de Ciencias Médicas, UNLP, Argentina.

## Sample Collection

At the end of the treatment, the animals were decapitated. Blood was collected using heparin (10 UI/mL) as anticoagulant in graduated ice-cold polypropylene tubes. Plasma samples were immediately prepared by centrifugation ( $4,000 \times g$ , 10 min) and then stored at  $-80$  °C until analyzed. Testes were rapidly excised, washed, weighed, and homogenized in ice-cold phosphate buffer 100 mM with 6 mM of EDTA pH 7.40 (3 ml of buffer to 1 g of tissue). Homogenates were stored at  $-80$  °C until assayed.

## Analytical Methods

Various biomarkers of oxidative stress and cell damage were measured in plasma and testicular interstitial cells.

Lipid peroxidation was assayed as authentic lipoperoxides (ROOHs) by the FOX assay [29]. ROOHs were expressed as nmol malondialdehyde (MDA)/mg protein. The sum of nitrates and nitrites ( $[\text{NO}_x]$ ) was measured as the main end-metabolite products of nitric oxide (NO) and peroxy nitrite anion ( $\text{ONOO}^-$ ) by the method of Miranda et al. [30]. Results were expressed as nmol of nitrites/mg protein. Protein carbonyls (nmol/mg of protein) were measured using the method of Reznick and Packer [31] as a biomarker of oxidative damage to proteins. We also measured parameters of the antioxidant defense system. The FRAP (ferric reducing ability of plasma) assay was determined by the method of Benzie and Strain [32] and the results were expressed as  $\mu\text{M}$  of equivalent TROLOX<sup>®</sup> or  $\alpha$ -tocopheryl acetate. Vitamin E ( $\alpha$ -tocopherol) was measured after extraction by the method of Buttriss and Diplock [33] using the HPLC technique of Bagnati et al. [34] which can detect and quantify both  $\alpha$ - and  $\gamma$ -tocopherols. Total glutathione (GSH) was measured by the method of Anderson and Meister [35] and expressed as  $\mu\text{mol}/\text{mg}$  of protein. Protein contents were determined according to the method of Bradford [36].

## Cell Culture and Treatment

Technical procedure for Leydig cell isolation was described in detail in a previous report [37]. Briefly, Leydig cells were removed from the interstitial space of testicular tissue by mechanical shaking with collagenase (Sigma Chem. Co., type IV) in a metabolic incubator, at 34 °C, according to Suescon et al. [38]. Cells were suspended in Krebs Ringer bicarbonate glucose (KRBG) solution (pH 7.4), examined for viability (90%) by trypan blue exclusion [39], and counted in a hemocytometer to adjust cell concentration. Aliquots of cell suspensions were subjected to protein determination by the micromethod of Bradford [36]. Preparations of interstitial cells comprised a high percentage of Leydig cells. The homogeneity of cell preparations was assessed by means of the observation of smears fixed in acetone and stained with H&E. Interstitial cell preparations consisted of Leydig cells (79%) and 21% of spermatids, spermatocytes and small cytoplasmic fragments.

## Functional Assay

The functional response of cultured interstitial cells was assayed by stimulation with chorionic gonadotrophin (h-CG, Ovusyn<sup>®</sup> from Syntex S.A., Buenos Aires, Argentina) 10 nM for 12 h. Cells were detached from the surface culture using mechanical harvesting, transferred to graduated polypropylene ice-cold tubes, and pelleted at  $600 \times g$  for 10 min. The pellet was washed twice with sonication buffer (65 mM Tris-HCl, pH 7.0, containing 10% sucrose)

and resuspended in the same medium. Cellular homogenates were prepared by sonication (three 30-s bursts at medium power in a Heat Systems Ultrasonic sonicator model W-220F from Plainview, NY). Crude extracts were centrifuged at  $2,000\times g$  for 15 min and the supernatants were used for hormone measurements.

### Immunoblotting

Cells were homogenized by sonication as described above in Tris/HCl/sucrose buffer with a 1% antiprotease cocktail (Sigma Chem. Co, Bs. As., Argentina), and 2% SDS. After heating the samples (20  $\mu$ g) at 95 °C for 3 min with 10% mercaptoethanol, aliquots were supplemented with 10% glycerol and 0.01% bromophenol blue and loaded on 12% SDS-PAGE mini-slabs (Mini protean II, Bio-Rad, Hercules, CA). Proteins were electrophoretically transferred overnight to PVDF membranes (Bio-Rad, Hercules, CA) using a transfer buffer containing 25 mM Tris, 190 mM glycine and 20% methanol. COX-2 protein levels were analyzed using a polyclonal antibody (Santa Cruz Biotechnology Inc., CA) while StAR protein was detected with a rabbit polyclonal antibody generated against amino acids 88–98 of the 30 kDa StAR protein prepared according to Clark et al. [17]. Blots were re-analyzed for  $\alpha$ -tubulin as the reference protein to control both loading and transfer processes. A polyclonal anti- $\alpha$ -tubulin antibody from Oncogene (San Diego, CA) was used. Immunocomplexes were revealed with peroxidase-labeled secondary antibodies from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Semi-quantitation of Western blots was performed using the GeneGenius gel documentation system and GeneTools software from Syngene (Cambridge, UK). Results were normalized to  $\alpha$ -tubulin signal and expressed as “fold change” relative to the control (basal assay) which was assigned a value of 1.

### Northern Blot Analysis

To determine StAR mRNA expression, Leydig cells were collected by centrifugation at 2 °C ( $3,000\times g$  for 10 min) and washed twice with PBS. Total RNA purification was performed with TRIzol reagent following the manufacturer's instructions (Gibco-BRL, Grand Island, NY). Quantification and purities were checked by determination of the absorbance ratio at 260/280 nm. Electrophoretic separation was performed in an agarose/formaldehyde gel with a linear gradient from 1 to 5%. Hybond-P membranes (Amersham, GE Healthcare Ltd., Buckinghamshire, UK) were used for blotting the slabs. Membranes were stripped with a buffer containing 15 mM NaCl, 15 mM Na citrate and 1% SDS (pH 7) for 40 min at 52 °C. StAR mRNA on the membrane was probed with biotin-labeled mouse StAR

cDNA and detected using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit from Pierce (Pierce Biotechnology Inc. Rockford, IL) following the manufacturer's instructions.

### PGF<sub>2 $\alpha$</sub> and PGE<sub>2</sub> Assays

For assessment of PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> contents in the incubation media of interstitial cells, samples were centrifuged at  $15,000\times g$  for 20 min at 2 °C. Supernatants were filtered through Millipore 0.2  $\mu$ m filters and concentrated by lyophilization in a Telstar Lyobeta freeze drying unit (Madrid, Spain). The residues were dissolved with HCl 2 N adjusting the pH to 3.5. These solutions were injected into a 200-mg C<sub>18</sub> reverse phase Sep-columns (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. Prostaglandins were determined using commercially available kits Prostaglandin F<sub>2 $\alpha$</sub>  EIA Kit and Prostaglandin E<sub>2</sub> 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% for both kits.

### Lipid Analysis

Total lipids were extracted by the method of Folch et al. [40]. The phospholipid fraction was separated from the Folch extracts by the micro-column chromatography method described elsewhere [41] and quantified as phosphorus content [42] after mineralization of an aliquot from the silicic acid partition. GLC of the FAME (fatty acid methyl esters) was performed as indicated in one of our previous papers [43] except that in this case we used a capillary column mounted on a Hewlett Packard HP 6890 Series GC System Plus (Avondale, PA) equipped with a terminal computer integrator and data station. The FAMES were identified by comparison of their relative retention times with authentic standards and mass distribution was calculated electronically by quantification of the peak areas. Eicosamonoenoic acid (20:1) was used as the internal standard. Cholesterol content was enzymatically measured according to Allain et al. [44] using a commercial kit from Wiener Lab. (Rosario, Argentina).

### Steroidogenic Enzyme Activities

Appropriate aliquots of supernatants were employed to determine 3- $\beta$ -hydroxysteroid-dehydrogenase (3 $\beta$ HSD) and 17- $\beta$ -hydroxysteroid-dehydrogenase (17 $\beta$ HSD) [EC 1.1.1.51] enzyme activities following the method of

Marugesan et al. [45]. Sonicated interstitial cells were centrifuged (10,000 × g, 15 min, 1–2 °C). Supernatants were mixed with charcoal in order to remove the endogenous steroids and the samples were again centrifuged (10,000 × g 15 min, 2 °C). The reaction conditions were defined in preliminary experiments, in which the 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 ( $\Delta^4$ -androstenedione or pregnenolone for 17 $\beta$ HSD and 3 $\beta$ HSD, respectively), 1 mM NADPH (17 $\beta$ HSD) or 0.2 mM NAD<sup>+</sup> (3 $\beta$ HSD), and 0.1 M phosphate buffer (pH 7.4). Mixtures were incubated under air in a spectrophotometric cell holder thermostated 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 control.

### Statistical Analysis

Results were analyzed by one way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Data were expressed as means  $\pm$  standard deviation (SD) of six independent determinations. They were considered different with respect to control data at the level of significance of \* $P < 0.05$  or \*\* $P < 0.01$ . In some of the Figures or Tables where multiple comparisons can be done we used different superscript letters to indicate statistical significances among results. Correlation and regression analyses and data plotting were automatically performed with the aid of Systat (version 12.0 for Windows) from SPSS Science (Chicago, IL) or Sigma Scientific Graphing Software (version 8.0) from Sigma Chem. Co. (St. Louis, MO).

## Results

### Influence of Treatments on Growth Parameters

The influence of the treatments on feeding parameters is shown in Table 1. Dimethoate treatment did not influence significantly the water consumption (approximately 15 mL/day), the final body weights, the rate of body weight gain, or the food efficiency ratio. Similar conclusions were obtained for the other treatments assayed (R, T or combined administrations; data not shown). We did not find absolute and relative testicular weight changes. Pesticide exposure—alone or in combination with the other drugs—did not affect the animal behavior. Also, no visible signs of toxicity and/or cholinergic effects were observed during the entire experimental period.

**Table 1** Main feeding parameters associated with experimental treatments

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

Values represent the means  $\pm$  SD ( $n = 8$ )

C control rats, D dimethoate-treated rats

<sup>a</sup> Food efficiency ratio = [body weight gain (g)/food intake (g)]  $\times 10^2$

<sup>b</sup> Relative testicular weight = testis weight (mg)/body mass (g)

### Testosterone Biosynthesis

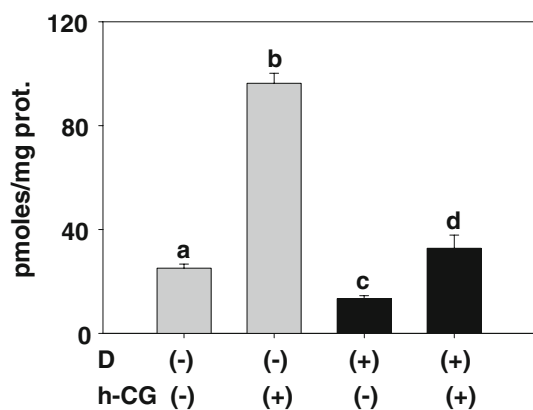
Plasmas from dimethoate (D)-treated rats contained less free and bound testosterone compared to control animals (Table 2). In samples from dosed rats, a decrease of approximately 20% was observed in both parameters whereas the ratio free/bound testosterone was not modified. We observed a more significant decrease in testosterone levels (approximately 50%) when sonicated interstitial cells were analyzed. Plasma estradiol concentration was decreased in D-treated rats by approximately 30%, while LH and FSH were increased by 58 and 76%, respectively (Table 2).

**Table 2** Hormone levels in plasma and interstitial cellular homogenates from control (C) or dimethoate-treated (D) rats

Determinations	C	D
Plasma		
Free testosterone (nM)	4.7 $\pm$ 0.2	3.9 $\pm$ 0.1**
Bound testosterone (nM)	22.2 $\pm$ 1.5	17.1 $\pm$ 3.0**
Free/bound testosterone $\times 10^3$	212 $\pm$ 15	228 $\pm$ 12
Estradiol (pg/mL)	15.1 $\pm$ 0.6	11.0 $\pm$ 0.4**
LH (mU/mL)	7.7 $\pm$ 0.2	12.2 $\pm$ 0.6**
FSH (mU/mL)	8.1 $\pm$ 0.4	14.3 $\pm$ 0.8**
Cellular homogenates		
Total testosterone (pmol/mg protein)	27.9 $\pm$ 1.3	14.2 $\pm$ 0.9**

Hormone levels were analyzed using RIA kits commercially available from Radim as indicated in the Experimental procedures. Data were expressed as the means  $\pm$  standard error of six independent determinations assayed in triplicate

Results significantly different to those of the corresponding control value are indicated with asterisks ( $P < 0.01$ )



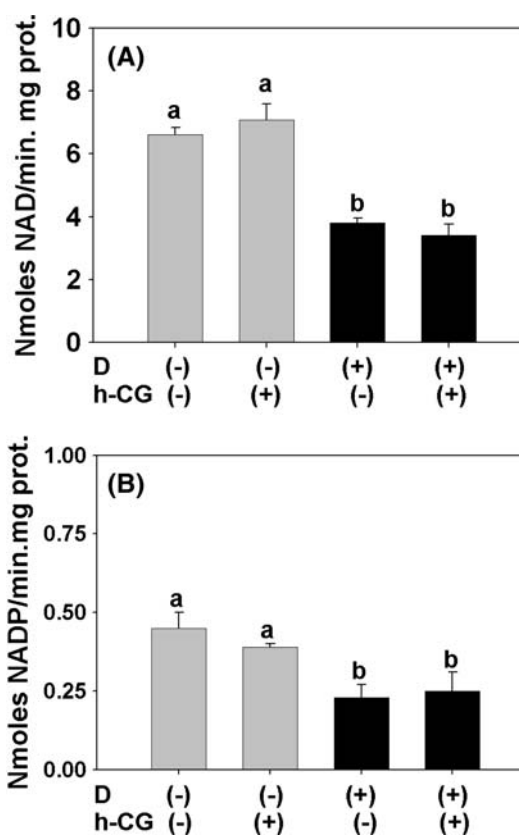
**Fig. 1** Testosterone production of interstitial cells isolated from control (gray bars) or dimethoate (D)-treated (black bars) rats incubated in the presence or not of h-CG 10 nM for 12 h. Results were the means  $\pm$  SD of six independent determinations performed by RIA as indicated in the “Experimental procedure”. Different superscript letters correspond to statistically significant differences at  $P < 0.01$

Interstitial cell cultures from C or D rats exhibited a very different behavior after 12-h stimulation with 10 nM human chorionic gonadotrophin (h-CG) (Fig. 1). Cells isolated from C rats released four times the basal testosterone production after overnight stimulation. In contrast, the cells isolated from D-treated rats exhibited significantly decreased basal levels and they only doubled the concentration of testosterone after the functional test with h-CG. Thus, we observed a 50% reduction in androgen production in h-CG-stimulated cells from D rats (Fig. 1).

Direct measurement of the two key androgenic enzyme activities which regulate testosterone biosynthesis were performed in supernatants from interstitial cell sonicates after a h-CG stimulation assay (Fig. 2). Both enzymes activities,  $3\beta$ - and  $17\beta$ -hydroxysteroid dehydrogenases ( $3\beta$ HSD and  $17\beta$ HSD, respectively) (Fig. 2A, B), were significantly lower (40–50%) after dimethoate treatment. Neither of the two enzymes exhibited a significant change after h-CG stimulation independent of the previous treatment of the animals and cells (Fig. 2).

#### Oxidative Stress Biomarkers

A condition of oxidative stress (OS) was clearly established in D-treated rats. This condition was characterized by a general failure of the antioxidant defense enzymes, a lower level of  $\alpha$ -tocopherol and increased concentrations of oxidative damage biomarkers in plasma (data not shown). In interstitial cells of D-treated rats, a significantly higher level of ROOHs which are reliable biomarkers of lipid oxidative damage (Fig. 3A). Levels of protein carbonyls

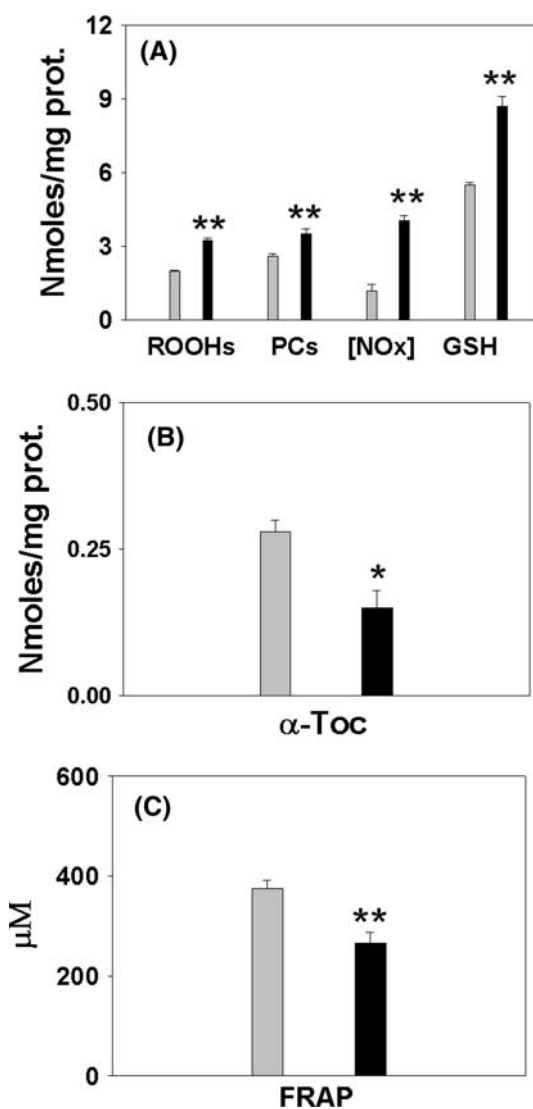


**Fig. 2** Steroidogenic enzyme activities in interstitial cells prepared from control (gray bars) or dimethoate (D)-treated (black bars) rats previously incubated or not with h-CG 10 nM for 12 h. Enzyme activities (A  $3\beta$ HSD, B  $17\beta$ HSD) were expressed as nmol of NAD or NADP/min mg protein and correspond to the means of six independent assays performed in duplicates  $\pm$  SD. See “Experimental procedure” for details. Different superscript letters correspond to statistically significant differences at  $P < 0.01$

and  $[\text{NO}_x]$  (nitrates + nitrites), which reflects the oxidative damage to proteins and the stimulation of NOS activity, respectively, were both increased by the administration of the pesticide (Fig. 3A). After dimethoate treatment, the total glutathione concentration increased (Fig. 3A) while  $\alpha$ -tocopherol decreased (Fig. 3B). The FRAP assay was performed in sonicated interstitial cells from C and D rats as a biomarker of the total antioxidant ability. In cells from D-treated rats, FRAP was lower by approximately 30% compared with control animals (Fig. 3C).

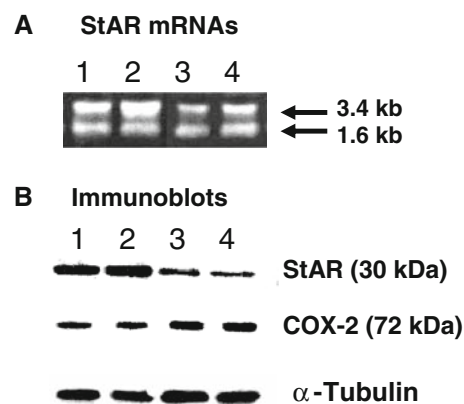
#### StAR and COX-2 Expression

The level of StAR mRNAs was estimated by Northern blotting. A representative result is shown in Fig. 4A. Lanes 1 and 2 correspond to control interstitial cells while lanes 3 and 4 to cells isolated from D-treated rats. Chorionic gonadotrophin was added to the cultures analyzed in lanes 2 and 4. The same scheme was followed when the



**Fig. 3** Oxidative stress biomarkers measured in interstitial cells from control (gray bars) or dimethoate-treated (black bars) rats. **A** Data for authentic lipoperoxides (ROOHs), protein carbonyls (PCs), nitrates + nitrites ([NO<sub>x</sub>]), and glutathione (GSH) levels. **B** α-tocopherol concentration (α-Toc), **C** the assay of the total antioxidant capacity (FRAP). Each value was expressed as the mean of six independent determinations ±SD. Asterisks indicate significant difference with respect to control value \**P* < 0.05 and \*\**P* < 0.01

expression of StAR or COX-2 proteins were tested by Western blotting (Fig. 4B). Relative quantification of the signals appeared in Fig. 5A and B. The h-CG stimulation provoked a significant increment of StAR at both transcriptional (Fig. 5A) and translational (Fig. 5B) levels. These increments were completely abolished by dimethoate treatment. Moreover, the pesticide evoked a reduction in the expression of StAR mRNAs, and also in its translation products (StAR protein). In contrast, dimethoate increased the biosynthesis of COX-2 protein at levels



**Fig. 4** Expression of mRNA StAR (**A**) and protein level for StAR and COX-2 (**B**) in interstitial cells. Lanes were: 1 control cells, 2 control stimulated with hCG 10 nM for 12 h, 3 D-treated cells, 4 D-treated cells stimulated with h-CG. Results are representative of six different incubations run in duplicate. See details in the “Experimental procedure” part

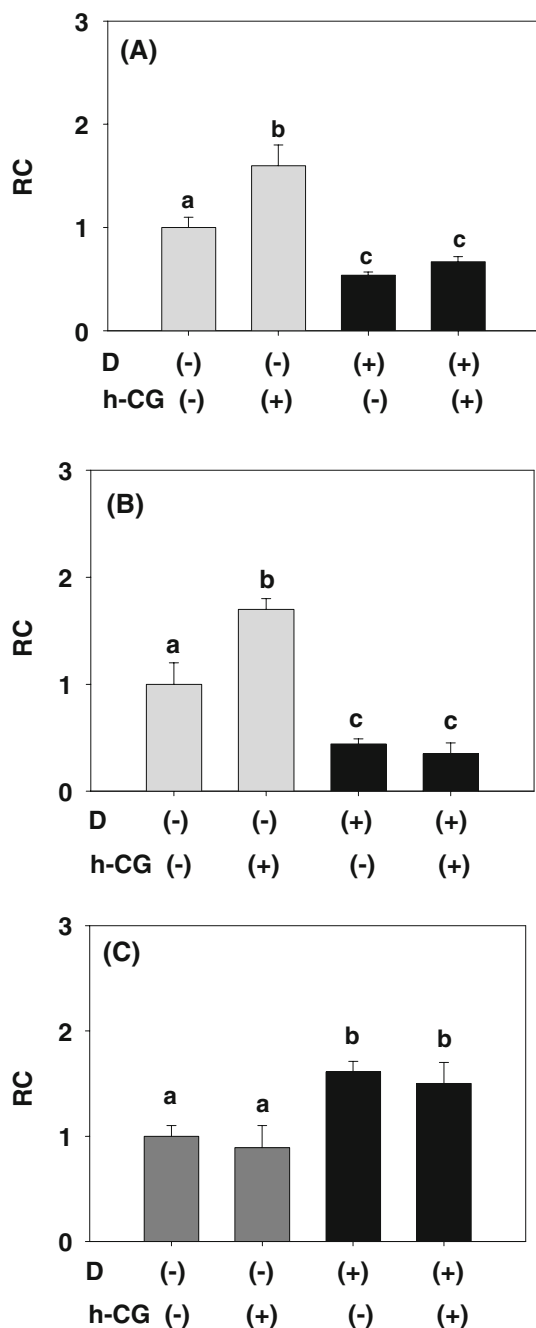
which were very similar to those observed in both h-CG stimulated and non-stimulated cells (Fig. 5C).

#### Arachidonate Metabolism and Cholesterol Level

The analysis of interstitial cell phospholipids demonstrated that the content of total inorganic phosphate per mg protein was unaffected by dimethoate treatment (data not shown). However, the pesticide significantly modified the fatty acyl composition of membrane phospholipids (Fig. 6). Saturated palmitic and stearic acids were increased while arachidonic, docosapenta-, and docosahexaenoic acids were diminished. Similar alterations were observed in neutral lipids (data not shown).

The total lipid extract from the mitochondrial fraction was also analyzed by the absolute content of cholesterol and arachidonic acid (Table 3) using a coupled enzymatic procedure with cholesterol oxidase and quantitative capillary gas-liquid chromatography, respectively. Clear correlations between the androgen production and the content of these lipids were observed (Fig. 7). Testosterone concentrations in whole cells were linearly and positively correlated with cholesterol content ( $r^2 = 0.92$ ) and arachidonate levels ( $r^2 = 0.98$ ) in mitochondrial fractions from interstitial cells.

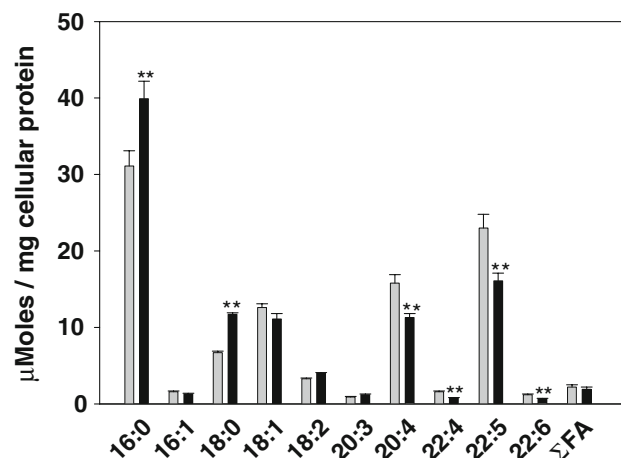
Cyclooxygenase-related metabolites of arachidonate were also studied in interstitial cells under basal or h-CG-stimulated conditions using EIA methods (Fig. 8). Prostaglandins F<sub>2α</sub>, and its precursor (PGE<sub>2</sub>), were both significantly higher in rats treated with dimethoate. Stimulation with h-CG provoked in both types of cellular cultures (control or D-treated) a slight—but not significant—change in the concentration of the prostaglandins analyzed compared with the corresponding reference culture (Fig. 8).



**Fig. 5** Relative changes (RC) of the expression of StAR mRNA (A), StAR protein (B) or COX-2 protein (C), quantified by densitometry using the GeneGenius gel documentation system and GeneTools software from Syngene (Cambridge, UK). Results were normalized to  $\alpha$ -tubulin signal and expressed as “fold change” relative to the control (basal assay) which was assigned a value of 1. Mean  $\pm$  SD. Different superscript letters correspond to statistically significant differences at  $P < 0.01$

#### Antioxidant and COX-Inhibitor Effects

The effects of the antioxidant TROLOX<sup>®</sup> or the COX inhibitor rofecoxib on various experimental parameters



**Fig. 6** Fatty acid composition of total phospholipids isolated from the mitochondrial fraction of interstitial cells prepared from control (gray bars) or dimethoate-treated (black bars) rats. Fatty acids were derivatized to methyl esters (FAMES) and analyzed by capillary gas-liquid chromatography under the conditions described in the “Experimental procedures”. FAMES were identified with authentic standards, quantified using eicosamonoenoic acid as internal standard and expressed as  $\mu$ mol of fatty acid per mg cellular protein (mean of six independent analyses  $\pm$  SD). Double asterisks indicate significant difference with respect to control value at  $P < 0.01$ .  $\Sigma$ FA corresponds to the sum of other C<sub>22</sub> and C<sub>24</sub> fatty acids not identified in the chromatogram

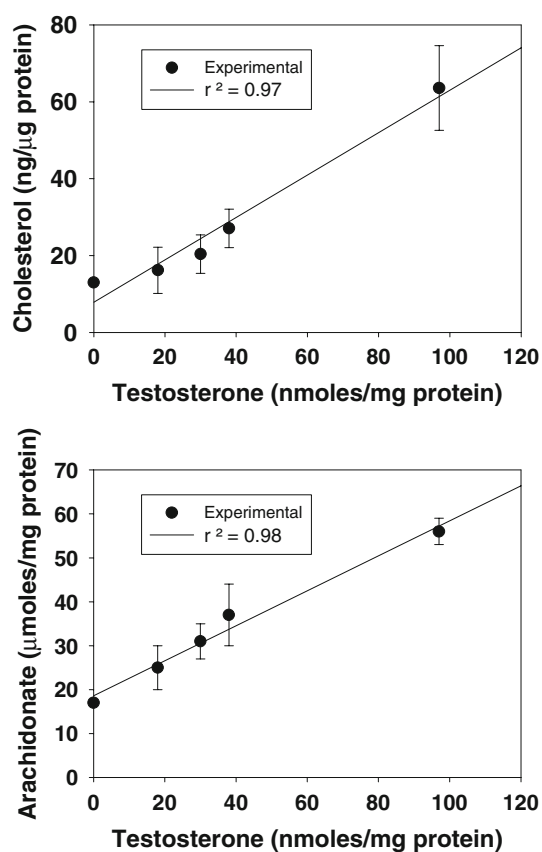
measured on dimethoate-treated rats appear in Tables 4 (absolute values) and 5 (relative changes). Drugs were administered alone or in combination at dosages that effectively inhibited lipid peroxidation and COX-2 activity. TROLOX<sup>®</sup> produced a strong decrease in the formation of D-induced ROOHs. However, the hormonal alterations evoked by dimethoate administration (essentially decreased testosterone biosynthesis and raised levels of the main gonadotrophin) were not normalized by simultaneous treatment with D and T. The levels of ARA were not affected by the treatment with the antioxidant alone. In addition, the decreased level of ARA observed after D administration was not restored when the pesticide was associated with T. Injection of R raised the level of ARA and at the same time significantly decreased the biosynthesis of PGF<sub>2 $\alpha$</sub> . Interestingly, testosterone concentration in both plasma and cellular homogenates was slightly (but significantly) higher while LH was lower under R treatment. Simultaneous administration of D and R normalized hormonal disturbances induced by D; notwithstanding the production of ROOHs still persistent and markedly elevated (Table 4). The ARA and PGF<sub>2 $\alpha$</sub>  D-induced changes were completely reverted by the opposite and proportional effects displayed by R (Table 5). When the three drugs were administered in combination (DTR) all the parameters of those of control rats were indistinguishable. From these



**Table 3** Cholesterol and arachidonic acid levels of total lipids from the mitochondrial fraction isolated from interstitial cells from control or dimethoate-treated rats

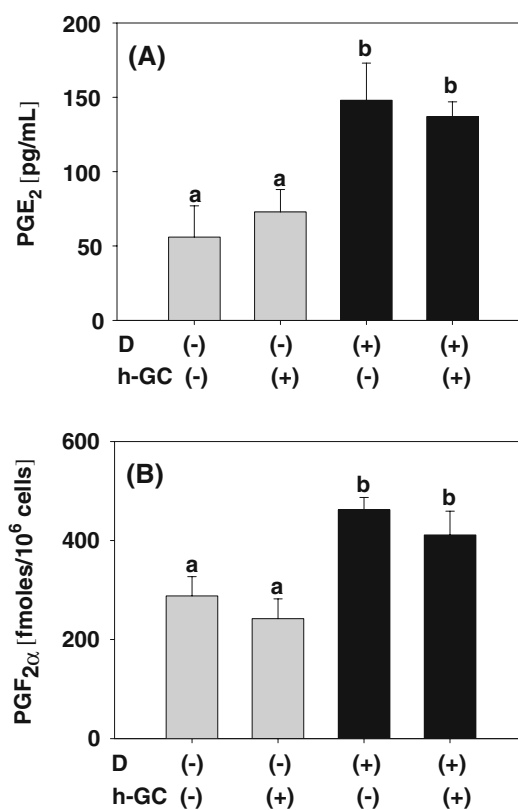
Cells	Control		Dimethoate	
Treatment	None	+h-CG	None	+h-CG
Lipids				
Cholesterol (ng/ $\mu$ g protein)	27.1 $\pm$ 5.0 <sup>a</sup>	63.6 $\pm$ 15.0 <sup>b</sup>	20.4 $\pm$ 5.2 <sup>c</sup>	16.2 $\pm$ 5.6 <sup>c</sup>
Arachidonic acid ( $\mu$ mol/mg protein)	36.2 $\pm$ 6.6 <sup>a</sup>	57.3 $\pm$ 3.2 <sup>b</sup>	25.5 $\pm$ 5.0 <sup>c</sup>	31.2 $\pm$ 4.3 <sup>c</sup>

Total lipids were extracted from the mitochondrial fraction of the interstitial cells prepared from control or dimethoate-treated rats after incubation with or without h-CG 10 nM for 12 h. Total lipid fractions were fractionated into neutral and polar lipids using silicic acid partition, and then analyzed for cholesterol and arachidonic acid levels using enzymatic and chromatographic methods as described in “[Experimental procedure](#)”. Results were expressed as the means of six independent determinations  $\pm$  1 SD of the mean. Different superscript letters correspond to statistically significant differences at  $P < 0.01$



**Fig. 7** Linear regressions and linear correlation coefficients ( $r^2$ ) for the relationship between cholesterol content of the mitochondrial fraction of interstitial cells and testosterone production (*upper panel*), or arachidonic acid level of the mitochondrial fraction of interstitial cells and testosterone production (*lower panel*). Data were obtained from basal or h-CG-stimulated cells from control or dimethoate-treated rats, and they were expressed as the means  $\pm$  SD of six independent measurements. Plots and  $r^2$  were automatically generated by means of the statistical software described in the “[Experimental procedure](#)”

results, the main conclusion obtained is that there is an inverse proportionality between the changes induced by each drug assayed (D, T or R) that results in neutralization



**Fig. 8** Prostaglandins (PG) production from interstitial cell suspension prepared from control (*gray bars*) or dimethoate (D)-treated (*black bars*) rats incubated in presence or absence of h-CG 10 nM for 12 h. PGE<sub>2</sub> (A) or PGF<sub>2 $\alpha$</sub>  (B) were analyzed by EIA as described in the “[Experimental procedure](#)” section. Each datum is the mean  $\pm$  SD of six independent assays. Different *superscript letters* correspond to statistically significant differences at  $P < 0.01$

of the effects when they are combined. For example, the decrease in testosterone or the increased biosynthesis of PGF<sub>2 $\alpha$</sub>  produced by D were almost exactly compensated by simultaneous treatment with R. Also, it is evident that the generation of ROOHs does not parallel the changes of ARA concentrations suggesting that other fatty acids (in addition to ARA) are involved in the generation of free radicals.

**Table 4** Main experimental parameters determined after dimethoate (D), TROLOX<sup>®</sup> (T), or rofecoxib (R) treatments to rats

Analytical parameters	Experimental groups						
	C	CT	CR	D	DT	DR	DTR
ARA ( $\mu\text{mol}/\text{mg}$ cell protein)	15.5 $\pm$ 0.7 <sup>a</sup>	16.2 $\pm$ 0.8 <sup>a</sup>	19.4 $\pm$ 0.9 <sup>b</sup>	9.8 $\pm$ 0.1 <sup>c</sup>	10.1 $\pm$ 0.5 <sup>c</sup>	15.3 $\pm$ 0.6 <sup>a</sup>	14.9 $\pm$ 0.8 <sup>a</sup>
PG F <sub>2<math>\alpha</math></sub> (fmol/10 <sup>6</sup> cells)	294.7 $\pm$ 11.9 <sup>a</sup>	319.3 $\pm$ 6.5 <sup>b</sup>	95.3 $\pm$ 8.5 <sup>c</sup>	454.4 $\pm$ 9.1 <sup>d</sup>	486.5 $\pm$ 12.3 <sup>c</sup>	301.9 $\pm$ 15.8 <sup>a</sup>	301.0 $\pm$ 9.6 <sup>a</sup>
ROOHs (nmol/mg protein)	3.1 $\pm$ 0.2 <sup>a</sup>	2.9 $\pm$ 0.3 <sup>a</sup>	3.4 $\pm$ 0.3 <sup>a</sup>	5.3 $\pm$ 0.1 <sup>b</sup>	3.6 $\pm$ 0.1 <sup>c</sup>	6.1 $\pm$ 0.8 <sup>b</sup>	3.7 $\pm$ 0.1 <sup>a</sup>
Hormones							
Plasma testosterone (nM)	5.0 $\pm$ 0.2 <sup>a</sup>	4.9 $\pm$ 0.1 <sup>a</sup>	5.9 $\pm$ 0.2 <sup>a</sup>	3.8 $\pm$ 0.1 <sup>b</sup>	4.0 $\pm$ 0.1 <sup>b</sup>	4.8 $\pm$ 0.1 <sup>a</sup>	5.1 $\pm$ 0.2 <sup>a</sup>
Cellular testosterone (pmol/mg protein)	26.6 $\pm$ 1.1 <sup>a</sup>	27.2 $\pm$ 0.8 <sup>a</sup>	32.1 $\pm$ 0.9 <sup>a</sup>	12.5 $\pm$ 1.0 <sup>b</sup>	14.3 $\pm$ 0.8 <sup>a</sup>	24.5 $\pm$ 0.8 <sup>a</sup>	27.1 $\pm$ 1.2 <sup>a</sup>
Plasma LH (mU/mL)	6.9 $\pm$ 0.2 <sup>a</sup>	7.0 $\pm$ 0.1 <sup>a</sup>	5.8 $\pm$ 0.2 <sup>a</sup>	15.1 $\pm$ 0.7 <sup>b</sup>	13.5 $\pm$ 0.5 <sup>b</sup>	7.3 $\pm$ 0.1 <sup>a</sup>	6.8 $\pm$ 0.2 <sup>a</sup>

Data are expressed as the means  $\pm$  SD (four independent determinations assayed in duplicate). C, control group (injected with vehicle alone). CT, CR and D were treated with TROLOX<sup>®</sup>, rofecoxib or dimethoate, respectively. DT, DR, and DTR received combined administration of the drugs. Determinations were performed as described in the “[Experimental procedure](#).” Statistical comparisons (ANOVA plus Tukey’s test) among experimental groups within a same row were indicated with different superscript letters

## Discussion

The increasing use for organophosphorus insecticides has introduced a serious and novel hazard for humans and livestock animals [22]. Dimethoate is one of the most used agrochemicals around the world. Experimental evidence indicates that it impairs fertility, suppresses libido, causes testicular degeneration, and deteriorates semen quality [22]. Despite its residual action, this drug is extensively used in horticulture as a systemic insecticide and acaricide for treating gardens, vineyards, and field crops, and it is also applied externally for the control of fly larvae in cattle [22]. Previous studies from other laboratories demonstrated its adverse effects on semen performance [22] which were attributed to the presence of the dimethyl group in the moiety [46]. In our experiments, the testes were macroscopically examined to find visible evidence of toxic effect(s). We observed considerable changes in appearance, volume, and consistence of both testes of treated rats that suggest tissue edema. However, we did not observe significant changes in absolute and/or relative weights such as those reported by Farag et al. [47]. This probably could be due to differences in animal species, time/route of treatment, and the dose of dimethoate administered. Since the wet weights were conserved, this finding may indicate loss of testicular-specific structural tissue. This was strongly supported by the aspects of the testes previously decapsulated, and it is in agreement with findings of Sayim et al. [48, 49] that revealed a dimethoate-dose depended histopathological features of rat testes. Alterations reported were characterized by moderate to severe seminiferous tubule degeneration as sloughing, atrophy, germ cell degeneration, and partial arrest of spermatogenesis [48, 49].

Dimethoate was also involved in hormonal unbalance. Thomas et al. [50] suggested that this pesticide could block

the uptake of steroid hormones at the interstitial cell receptor level. However, other research has previously proposed that dimethoate exerts a direct cytotoxic action not associated with alterations in LH, FSH, or other hormonal regulators implicated in androgen biosynthesis [51, 52]. The present work demonstrate that low doses of dimethoate administered i.p. chronically are able to produce significant alterations in the levels of circulating gonadotrophin hormones which raise in response to a decrease testosterone production. The LH and FSH increases may have important consequences in the differentiation status of Leydig cells. In fact, previous studies demonstrated that this chronic stimulation is closely related to increased cancer degeneration [8]. The estradiol level, which reflects testosterone metabolism, is also significantly reduced after dimethoate treatment. Our results agree with an early investigation by Afifi et al. [22] who reported a decrease testosterone level of rats dosed orally with dimethoate for 2 months. We are unaware of any other studies that have examined in more detail the hormonal profile after dimethoate intoxication.

Oxidative stress is a well-known consequence of intoxication with environmental contaminants. This condition was repeatedly observed under metal overload [14, 53–56] or after intoxication with agrochemicals [20, 57, 58]. Some of the detrimental effects produced by pesticides in the male reproductive system were explained as a consequence of the exacerbated free radical production caused by these drugs [59]. It was proposed that most pesticides may act as pseudosubstrates for the generation of free radicals, which in turn, can damage P450 enzymes [6]. However, this suggestion is not accepted for all researchers since antioxidant supplementation failed to restore testosterone biosynthesis after intoxication by, for example, octylphenols [57]. Our results indicate that cultures of interstitial cells isolated from D-treated rats have a clear

oxidative stress condition hallmarked by a general loss of the antioxidant capacity and elevation of biomarkers of OS damage. Interestingly, we observed an increased amount of the water-soluble antioxidant glutathione that could be ascribed to a compensatory mechanism of scavenging against the increased production of free radicals induced by dimethoate. This explanation is in agreement with previous experimental evidence indicating that  $\gamma$ -glutamyl-cysteinyl synthetase—which controls the biosynthesis of glutathione—is induced by a prolonged oxidative stress condition [60, 61]. At the same time, the decreased level of  $\alpha$ -tocopherol could be the consequence of its increased rate of consumption within the lipophilic compartment of the interstitial cells. The loss of the lipid-soluble antioxidant capacity correlates well with the observed increase in the ROOHs production and the lower FRAP values. These findings led us to the idea that the testosterone level may be restored by antioxidant treatment. However, in agreement with the results reported by Muroño et al. [57] (for the octylphenol inhibitory effect on testosterone biosynthesis) we did not find any positive effect caused by concomitant administration of D and T (Tables 4, 5).

From this scenario, a first explanation for the decreased testosterone production could be proposed. Biosynthesis of this androgen in Leydig cells depends on the activities of two key enzymes ( $3\beta$ HSD and  $17\beta$ HSD) which were inhibited in our experimental system in a clear correlation with the observed decrease in both the production of testosterone by Leydig cells and the plasma testosterone level. During the last years, free radical production has been linked to its possible role in reducing steroidogenesis [62–65]. In addition, studies from Murugesan et al. [20, 45, 66] and Kostic et al. [67] suggested that androgenic dehydrogenases could be significantly inhibited by free radical overproduction. Thus, we think that—as judged by the normalization of ROOHs—OS induced by dimethoate treatment can be reverted by T administration; however, this fact may not contribute at least crucially to the loss of the androgenic capacity via  $3\beta$ - and  $17\beta$ -dehydrogenase inhibition. These results support the assumption that D does not act as a pseudosubstrate that binds to the steroid-binding site of P450 enzymes (P450<sub>scc</sub> and P450<sub>c17</sub>) disrupting hydroxylation and leading to electron leakage and free radical production [57]. Also, it is unlikely that OS condition affects significantly the availability of NADPH that is required for the normal hydroxylation of steroids during P450 pathway for testosterone biosynthesis.

Another question concerning OS and steroidogenic activity can be discussed. It is well known that lipids can be destroyed by free radical attack. In particular, lipid peroxidation is now considered to be the main mechanism by which oxygen- or nitrogen-derived radicals can cause damage leading to impaired normal cellular function in

testis [68]. Mammalian spermatozoa, being rich in polyunsaturated fatty acids (PUFAs) are more susceptible to oxidative damage resulting in sperm deterioration [6, 49]. Similar consequences can be assumed for Leydig cells which are also very rich in PUFAs [69]. Induction of OS by pesticides has been a focus of research over the last decade as a possible mechanism for infertility [6, 70]. We observe that after intoxication with dimethoate a significant loss of PUFAs occurred in interstitial cells, specially arachidonic, docosapenta- and hexaenoic fatty acids (Fig. 6). C<sub>22</sub> PUFAs are involved in spermatogenesis [71] while arachidonic acid (ARA) is directly linked to the androgenic activity of Leydig cells [16, 19]. Thus, it is possible that chronic OS may be an important mechanism for the arachidonate-dependent loss of steroidogenic ability since this fatty acid is essential for the biological activity of the steroidogenic acute regulatory protein (StAR) [72]. ARA level in Leydig cells is controlled by a hormone-regulated mechanism which involves an acyl-CoA synthetase (ACS4) and a mitochondrial acyl-CoA thioesterase (Acot2). Recent findings have demonstrated that, in steroidogenic cells, ARA release does not operate through the activation of the phospholipase A<sub>2</sub> pathway [73]. Experimental evidence supports the idea that ARA is released from the cholesteryl-ester pool and immediately converted into arachidonoyl-CoA by ACS4 [19]. This thioester binds to the acyl-CoA binding protein (DBI), which in turn, binds to the translocator protein (TSPO) located in the outer mitochondrial membrane. This would possibly facilitate a direct transference of arachidonoyl-CoA into the mitochondria [72]. In addition, ARA is transformed into lipoxygenated products which induce the transcription of StAR gene [72]. Thus, the conversion of cholesterol to testosterone is limited by the transport of cholesterol from the outer to the inner mitochondrial membrane. This event is controlled by StAR activity [21] and also by the ARA level [73, 74]. We have demonstrated that both ARA and cholesterol concentration are decreased in cells isolated from dimethoate-treated rats. Reduction of ARA, which in turn leads to the reduction of mitochondrial cholesterol content, could be produced by oxidative destruction of PUFAs due to D-induced OS, by an increased utilization via the COX-2 pathway, or both. Previous studies with rat mitochondrial fractions subjected to peroxidative conditions support the assumption that PUFA content can be effectively depleted by overproduction of free radicals [75]. In addition, recent studies from our laboratory indicated that plasmalogen subfraction is particularly enriched in ARA and very sensible to oxidative damage [76]. In addition, another pathway for ARA metabolism which is very active in interstitial cells, is the cyclooxygenase route that converts the precursor ARA into prostaglandins G<sub>2</sub>, H<sub>2</sub>, E<sub>2</sub>, D<sub>2</sub> and F<sub>2 $\alpha$</sub>  [77]. Cyclooxygenase isoenzymes types

**Table 5** Statistical comparisons and percent changes among experimental parameters determined after dimethoate (D), TROLOX® (T), or rofecoxib (R) treatments to rats

Parameters	Groups					
	CT	CR	D	DT	DR	DTR
ARA	4.5 ± 0.1 n.s.	25.2 ± 1.2*	-36.7 ± 1.6**	-34.8 ± 1.1**	-1.3 ± 0.1 n.s.	-3.9 ± 0.2 n.s.
PGF <sub>2α</sub>	8.3 ± 0.2 n.s.	-67.7 ± 2.1**	54.2 ± 1.5**	65.1 ± 2.4**	2.4 ± 0.2 n.s.	2.1 ± 0.3 n.s.
ROOHs	-6.5 ± 0.2 n.s.	9.7 ± 0.3 n.s.	70.9 ± 1.9**	16.1 ± 0.5*	96.7 ± 2.1**	19.4 ± 0.4*
Te (plasma)	-2.0 ± 0.1 n.s.	18.0 ± 0.4*	-24.0 ± 0.6**	-20.0 ± 0.5*	-4.0 ± 0.1 n.s.	2.0 ± 0.1 n.s.
Te (cells)	2.2 ± 0.1 n.s.	17.1 ± 0.3*	-53.0 ± 2.1**	-46.2 ± 1.1**	-7.9 ± 0.3 n.s.	1.9 ± 0.1 n.s.
LH (plasma)	1.4 ± 0.1 n.s.	-15.9 ± 0.5*	118.8 ± 4.6**	95.6 ± 3.1**	5.8 ± 0.2 n.s.	-1.4 ± 0.1 n.s.

Data were obtained from Table 4 and assayed by ANOVA plus Tukey's test. Percentage changes (expressed as means ± SD) for a determined treatment were calculated as  $[X - C] \times 100/C$ , being X and C the tested and control paired data, respectively, within each group under comparison

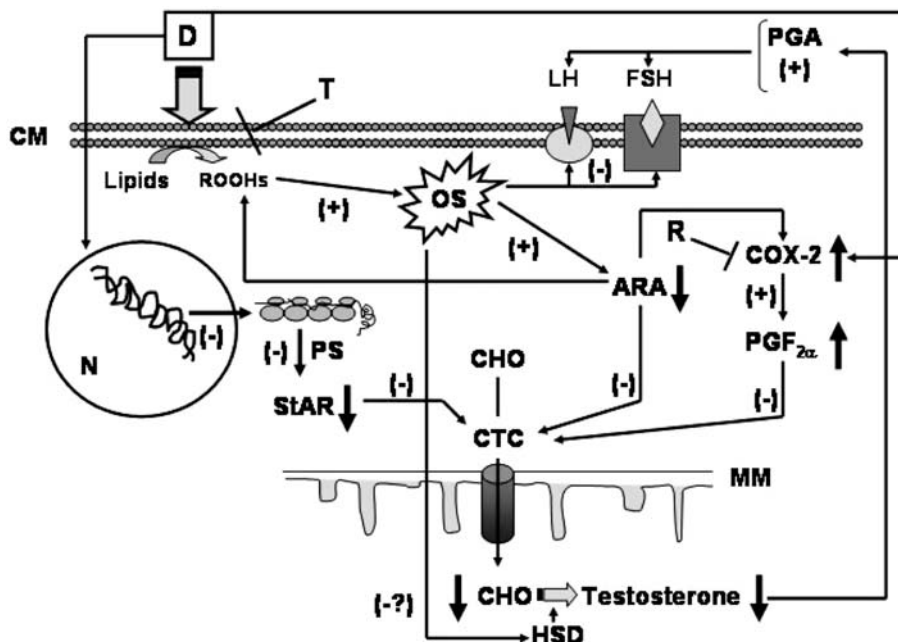
ARA arachidonic acid, PGF<sub>2α</sub> prostaglandin F<sub>2α</sub>, ROOHs authentic hydroperoxides, Te testosterone

Statistical significances were indicated by asterisks (\* $P < 0.05$  and \*\* $P < 0.01$ ), or with "n.s." (non-significant difference respect to the corresponding control group)

I (COX-1) and II (COX-2) are key enzymes that function coordinately and are controlled differentially by regulating the amount of ARA and lipid peroxides [78]. Previous studies from other laboratories suggested that COX-1 and COX-2 may not be important for male reproductive performance, at least in mice [77]. However, this early general view is being challenged by recent findings. COX-2 is overexpressed in human testis from men with impaired spermatogenesis, infertility, or degenerative disorders [79, 80]. Moreover, PGF<sub>2α</sub> has been reported to significantly decrease StAR expression in porcine, rat, and human tissues [81–84]. In addition, Frungieri et al. [74] demonstrated that PGF<sub>2α</sub> inhibition of h-CG-induced testosterone production in Leydig cells is accompanied by the down-regulation of both StAR and 17βHSD. Moreover, we observed elevated levels of trophic hormones in peripheral blood which failed to restore the androgenic capacity. This was in agreement with a previously demonstrated blockage of PGF<sub>2α</sub> of the LH- or FSH-stimulatory pathway [85]. In vitro studies using MA-10 mouse Leydig cells further proved that inhibition of COX-2 activity significantly increased StAR protein expression and steroid production [86]. Our results strongly suggest that COX-2 is implicated in the inhibition of testosterone biosynthesis by dimethoate. We observed a significant increase in the level of COX-2 protein in cells isolated from dosed animals which correlated with the higher concentrations of both PGE<sub>2</sub> and PGF<sub>2α</sub>. Concomitantly, StAR protein is diminished at both the gene expression and protein levels. These results also agree with those of Arakane et al. [87] who reported an inhibition of StAR gene expression by PGF<sub>2α</sub>. Concerning the role of COX-2 in testosterone production, we demonstrated that simultaneous administration of rofecoxib and dimethoate normalizes the hormonal levels to control values although the COX inhibitor was not able to modify the

ROOHs production (Tables 4, 5). From these results, we assume that the main target of dimethoate effect on steroidogenic route is COX-2 activity that leads to the overproduction of PGE<sub>2</sub> and F<sub>2α</sub> and subsequent inhibition of the StAR regulatory mechanism.

The status of androgenesis is usually tested (in vivo or in vitro) by the commonly accepted method of h-CG stimulation followed by the measurement of the testosterone production [12]. Interestingly, in our experimental system, the functional assay of stimulation with physiological concentration of h-CG does not normalize the changes induced by dimethoate, suggesting that the pesticide could act independently of gonadotrophin receptor-mediated events. This fact should be considered as another mechanism by which dimethoate can depress the pathway for androgen production. It remains to be explored whether this inhibitory response was initially triggered by overproduction of free radicals, and to what extent the overall effect can be attributed to the other(s) (synergistic) mechanism(s) involved. For example, previous studies by Diemer et al. [85] demonstrated that free radicals per se are able to inhibit StAR activity. However, results from Tables 4 and 5 suggest that OS may not have a crucial role in blocking StAR signal cascade directly or indirectly via gonadotrophin receptors. Whenever this crosstalk may (or not) occur, it seems quite possible that the ARA level was not (at least not entirely) depleted as a consequence of the oxidative stress condition since administration of D or R decrease and increase, respectively, the level of the acid in a clear correspondence with the COX-2 activity changes (Tables 4, 5). Thus, the increased production of PGE<sub>2</sub> and PGF<sub>2α</sub> should be produced—as observed in our experiments—through an important stimulation of the COX-2 activity which is inversely associated to the concentration of its substrate (ARA). As a consequence, StAR activity



**Fig. 9** Effects of chronic dimethoate intoxication on the steroidogenic status in rat testis. Dimethoate (*D*) evokes a general failure in the antioxidant system and exacerbates the production of lipoperoxides (*ROOHs*) which leads to an oxidative stress condition (*OS*). This effect is effectively blocked by administration of TROLOX® (*T*). The arachidonic acid (*ARA*) level decreases by conversion into peroxides principally due to exacerbated COX-2 activity rather than the destruction by free radicals. This condition diminishes the efficiency of the cholesterol transport complex (*CTC*) located at the mitochondrial membrane (*MM*). *ARA* is converted by the induced cyclooxygenase-2 (*COX-2*) into prostaglandin  $F_{2\alpha}$  (*PGF\_{2\alpha}*) that inhibits the activity of the *CTC*. Rofecoxib (*R*) blocks the activity

of COX-2 and prevents the deleterious effect(s) of dimethoate (*D*). Administration of *D* also reduces the expression of the gene which encodes the steroid acute regulatory protein (*StAR*) and its translation into active protein. The decreased production of *StAR* further inhibits the input of cholesterol (*CHO*) by the mitochondria. All these effects act in concert to decrease the availability of *CHO* and its conversion into testosterone. Additional effects should be exerted on the activities of the 3 $\beta$ - and 17 $\beta$ -hydroxysteroid dehydrogenases (*HSD*), and on the signal transduction of LH and FSH which are increased by the pituitary–gonadal axis (*PGA*) in response to the decreased production of testosterone. *CM* cytoplasmic membrane, *N* nucleus, *PS* protein synthesis

would be inhibited indirectly by either a decreased availability of *ARA* or an increased concentration of  $PGF_{2\alpha}$ . As reported previously, steroidogenesis is sensitive to small changes in *StAR* protein expression [21]. This question is important since *StAR* plays a key role in steroid production by gonads as well as in the steroidogenesis of adrenal glands. As a result, a disruption in *StAR* protein expression may impair more than just fertility. The biosynthesis of mineralo- and glucocorticoids may affect carbohydrate metabolism, immune system, and water balance. Such a general disturb may underline many of the toxic effects of environmental pollutants [21, 88].

As a conclusion, we propose that dimethoate inhibits testosterone biosynthesis by multiple mechanisms of action (Fig. 9). Chronic intoxication, even at the low doses used in our experiments, provokes an oxidative stress which in turn raises the level of lipoperoxides and the activity of COX-2. As a consequence, a decreased rate of the *StAR*-stimulated cholesterol transport into the mitochondria was observed. Dimethoate treatment also blocks the activities

of the hydroxysteroid dehydrogenases and impairs the LH- or FSH-dependent signal for stimulation of androgen biosynthesis by a mechanism(s) that do not depend crucially on the *ROOHs* level. At the same time, increased COX-2 protein decreases *StAR* transcription and translation. The subsequent elevation of  $PGF_{2\alpha}$  further inhibits *StAR* protein action decreasing both the availability of cholesterol and the rate of testosterone biosynthesis probably by inhibition of the 17 $\beta$ HSD mRNA transcription. The final biological effect suggests that the androgenesis status could provide a highly sensitive test for estimating the damage exerted by residual agrochemicals in chronically exposed populations. These conclusions should stimulate further research concerning the androgen functionality and the hormone level of men involuntarily exposed to dimethoate or others organophosphorus compounds. This fact also raised concern about the generality of the toxic action(s) of this kind of drugs on both the reproductive functions and the other metabolic pathways controlled by steroid hormones.

**Acknowledgments** This study was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. We would like to thank Mrs. Agustina Zardis de Cobeñas, Eva Illara de Bozzolo, and Norma Cristalli for their excellent technical assistance.

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