



Melatonin prevents damage elicited by the organophosphorous pesticide diazinon on the mouse testis

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ARTICLE INFO

Article history:

Received 2 August 2007

Received in revised form

18 April 2008

Accepted 27 April 2008

Available online 18 June 2008

Keywords:

Diazinon

Melatonin

Testis

SOD

Testosterone

Oxygen radicals

ABSTRACT

Organophosphates like *O,O*-diethyl *O*-2-isopropyl-6-methyl pyrimidinyl-4-g-1-phosphorothioate (diazinon) are pesticides used worldwide, which can affect both animals and man even after a single exposure. Whereas their toxicity is due to acetylcholinesterase inhibition, their secondary toxic effects have been related to free oxygen radicals. This study evaluates the effects of a single dose of diazinon and melatonin—a powerful antioxidant—on plasmatic acetylcholinesterase activity and testis histopathology in adult mice 1 and 32 days post-treatment. Diazinon diminished the plasma acetylcholinesterase activity on day 1 post-treatment, although testosterone levels remained unaffected. Morphometrical analysis showed a decrease in seminiferous epithelium height (days 1 and 32), whereas an increase in testicular superoxide dismutase (SOD) activity was detected (day 32). Melatonin pretreatment prevented every alteration induced by diazinon, except the diminution of acetylcholinesterase plasmatic activity. Testicular damage might be due to elevated concentrations of free oxygen radicals released upon diazinon exposure, inducing alterations in the DNA and promoting local apoptosis; however, antioxidant pretreatment with melatonin prevents or diminishes this damage.

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1. Introduction

Organophosphorous (OP) pesticides are among the chemical compounds most commonly used to control agricultural plagues. Their mechanism of action is based on the inhibition of acetylcholinesterase activity through covalent binding to its serine residues, thus producing a detention of the nerve impulses that leads to death (Booth and McDonald, 1988). Amongst the most frequently used OP insecticides, *O,O*-diethyl *O*-2-isopropyl-6-methyl pyrimidinyl-4-g-1-phosphorothioate (diazinon), a chemical synthetic substance, is used worldwide to eliminate crop and cattle plagues, as well as in household pest control (ATSDR, 1997). Diazinon can be absorbed through the digestive system, the skin, or via the respiratory tract when inhaled. Although it is mainly eliminated by the kidney, microsomal enzymes in the liver oxidize diazinon producing more potent acetylcholinesterase inhibitors, such as diazoxon, hydroxydiazoxon and hydroxydiazinon (WHO, 1998).

Infertility in humans has been correlated with exposure to pesticides (Strohmer et al., 1993). It has been observed that the spermatocytogenesis and spermiogenesis are the spermatogenic stages most susceptible to environmental toxicants, therefore

being able—directly or indirectly—to permanently damage the testis and affect male reproduction (Björge et al., 1996). Pesticides also produce endocrine alterations, leading to changes in diverse reproductive parameters. It is known that quinalphos affects the union of androgens and/or estrogens to their receptors (Sarkar et al., 2000). On the other hand, lindane induces a decrease in plasmatic testosterone levels as a consequence of alterations in steroidogenesis probably through adverse effects on the Leydig cells located in the testicular interstices (Ronco et al., 2001). These cells are the main producers of testosterone, a hormone that contributes to normal spermatogenesis, normal Sertoli cells function and to the development of the secondary glands of male reproductive system (Dadoune and Demoulin, 1993). Moreover, the OP chlorpyrifos causes a significant reduction in the activity of superoxide dismutase (SOD), an enzymatic defensive system which permits the dismutation of the superoxide ion into hydrogen peroxide, the accumulation of which is avoided by the catalase/glutathion peroxidase (CAT/GPX) system by transforming it into water and molecular oxygen or oxidized glutathione, respectively (Pierrefiche and Laborit, 1995). When these systems fail or get surpassed, there is an overproduction of superoxide ions and hydrogen peroxide which, when not completely detoxified, give rise to the highly toxic hydroxyl radical.

5-Methoxy-*N*-acetyltryptamine (melatonin) is an indol synthesized from tryptophane in various organs such as the pineal gland, retina, intestine, bone marrow cells and skin (Tan et al., 2003). It is

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very lipophilic and crosses all lipidic membranes, including the cytoplasmic membrane. As a result, melatonin reaches the cytosol of all cells; thereby its function is not restricted solely to a certain group (Karbownik and Reiter, 2000). At the molecular level, its participation in steroidogenesis has been demonstrated (Vera et al., 1997). The finding of mel 1a receptors in Leydig cells *in vitro* has been associated with melatonin's capacity to block hCG stimulation and production of necessary cAMP for the synthesis of enzymes which participate in the transformation of cholesterol into testosterone, like the 17- and the 3-hydroxysteroid dehydrogenase. Likewise, melatonin diminishes the expression of the steroidogenic acute regulation (StAR) protein, which is essential for steroidogenesis (Frungieri et al., 2005).

Melatonin can reduce oxidative stress by stimulating important enzymes such as SOD and glutathion peroxidase (GSH-Px) (Onur et al., 2004). Taking into consideration that diazinon-induced oxidative stress and reactive oxygen species (ROS) generation has been demonstrated after acute toxicity in an *in vivo* murine model (Teimouri et al., 2006; Giordano et al., 2007; Sutcu et al., 2007), and although the protective mechanism of melatonin in most cases has not been completely clarified (Reiter et al., 1995; Reiter, 2002), it is interesting to elucidate if the antioxidant molecule melatonin could prevent the toxic effects provoked by an organophosphate pesticide like diazinon under conditions of acute toxicity on the testicular tissue. Additionally, it is important to establish if testosterone production by Leydig cells persists without alteration at late times after OP exposure.

2. Materials and methods

2.1. Chemicals

Melatonin (99.9% purity) was kindly supplied by Arama Laboratories (Santiago, Chile) and a commercial formulation of diazinon (Diazyl[®], 60% active ingredient) was purchased from Eximerk Laboratories (Santiago, Chile).

2.2. Animals

Seventy-two adult CF1 mice of 12 weeks of age were housed under standard lighting conditions (12 light:12 dark), allowed access to food and water *ad libitum* and maintained between 18 and 20 °C. All animal studies were conducted in accordance with the principles and procedures outlined by the Bioethics Committee of the School of Medicine, University of Chile. Mice were separated in 12 cages of six individuals each and injected with 200 µL of solution. Male mice from the control group (Group 1) were injected intraperitoneally (ip) with a vehicle (ethanol 3% in NaCl 0.9%). The experimental groups were injected with diazinon (dz) diluted in vehicle in the following doses: $\frac{1}{3}$ of the LD₅₀ (Group 2), $\frac{2}{3}$ of the LD₅₀ (Group 3), with melatonin (mlt) (Group 4), mlt–dz $\frac{1}{3}$ of the LD₅₀ (Group 5), and mlt–dz $\frac{2}{3}$ of LD₅₀ (Group 6). All groups were evaluated on days 1 and 32 post-injection (pi). The groups administered with both melatonin and diazinon received melatonin 30 min prior to diazinon. The chosen dose of melatonin was 10 mg/kg of body weight (Gultekin et al., 2001), and the lethal dose 50 (LD₅₀) for diazinon was previously determined on 65 mg/kg of body weight, in our laboratory (data not shown).

2.3. Testicular histology and evaluation

After sacrificing the animals by overdose of anesthesia, the left testis was extracted, fixed in Bouin's fixative and subjected to routine histological techniques, obtaining 5-µm-thick cross-sections to perform the morphometric analysis of a total of 500 tubules per group, which consisted of measuring the height of the seminiferous epithelium, tubular diameter and testicular interstice–tubule ratio. Samples were stained with the hematoxylin–periodic acid–Schiff (PAS) method (McManus, 1946). The tissues were observed under a light microscope and photographed with a digital camera (Sony, model DSC-P71, Japan) at 400 ×, and images were processed and analyzed with the Image Tools 3.0 software (available online at <http://ddsdx.uthscsa.edu/dig/itdsc.html>).

2.4. Determination of plasmatic cholinesterase activity

Plasma was obtained by cardiac puncture at the moment of euthanasia, using a heparinized tuberculin syringe. Enzymatic activity was determined using the Ellman's method for cholinesterases, involving the substrate acetylthiocholine. Its hydrolysis product, thiocholine, reacts with dithiobis-2-nitrobenzoate (DTNB) producing 5-thionitrobenzoate, an ion that absorbs strongly at 412 nm (Ellman et al., 1961).

2.5. Plasmatic testosterone determination

This assay was performed in heparinized plasma through direct radioimmunoassay using a commercial kit (Coat-A-Count Total Testosterone, Diagnostic Products Co., Los Angeles, CA), with a minimum sensibility of 0.14 nM and an interassay coefficient with variation of 11%. The Coat-A-Count kit consists in a solid-phase radioimmunoassay based on a specific antibody for testosterone that is immobilized on the wall of a polypropylene tube. The I¹²⁵-tagged testosterone competes with testosterone from the plasma sample for the binding spaces of the antibody. After leaving the tube to decant, the antibody-attached testosterone is measured using a gamma counter.

2.6. Determination of superoxide dismutase (SOD) activity

After surgical extraction of the right testis, it was dissected out, cleaned with PBS pH 7.3 at 4 °C, and weighed; then, it was placed in a volume of PBS 45 times bigger than the testicular tissue weight. Each testis was homogenized separately in a T-line homogenizer laboratory stirrer (model 106) with 25 strokes and centrifuged at 33,000g at 4 °C during 45 min in a Sorvall Dupont RC-5B ultracentrifuge, and followed by supernatant freezing at –20 °C until the moment of the determinations. Total SOD activity was determined on the basis of the epinephrine–adrenochrome autoxidation (Misra and Fridovich, 1977). The method is based on the SOD capacity to inhibit epinephrine autoxidation and subsequent adrenochrome formation. Epinephrine conversion has a maximum absorption peak at 480 nm, and this was read in a Hewlett Packard 8452 spectrophotometer. Results were expressed in U units (mg/mL proteins) (Misra and Fridovich, 1977).

2.7. Statistical analysis

All data were analysed using the non-parametric Kruskal–Wallis test and the *post-hoc* test of Dunn. Results are presented as the mean ± standard deviation (SD), except for the testosterone assay, where the results are presented as the mean ± standard error of the mean (SEM). *p* values smaller than 0.05 (*p* < 0.05) were considered statistically significant.

3. Results

3.1. Diazinon and melatonin effect on plasmatic acetylcholinesterase

On day 1 pi, treatment with diazinon provoked a significant decrease in plasma acetylcholinesterase levels in all treatment groups that received the pesticide when compared to control, even regardless of the previous treatment with melatonin (*p* < 0.05, control: 0.90 ± 0.42 µmol/min/mL). On day 32 pi, detected levels of cholinesterase in all groups were similar to control values. The control group showed a cholinesterase concentration of 1.27 ± 0.73 µmol/min/mL (Fig. 1).

3.2. Diazinon and melatonin effect on seminiferous epithelium height

On day 1 pi, dz $\frac{2}{3}$ LD₅₀ produced a significant decrease in epithelial height (34 ± 5) compared to control (47 ± 4 µm) (Figs. 2 and 3). The group treated with dz $\frac{1}{3}$ LD₅₀ did not present significant changes (45 ± 3 µm). Similarly, the group treated with mlt+dz $\frac{1}{3}$ and mlt+dz $\frac{2}{3}$ LD₅₀ did not show changes in epithelial height (43 ± 4 µm) with respect to control. Nevertheless, the epithelial height of the mlt+dz $\frac{2}{3}$ LD₅₀ group was significantly higher than that observed in the group treated with only dz $\frac{2}{3}$ LD₅₀ (Fig. 4). At day 32 pi, the groups treated with dz $\frac{1}{3}$ LD₅₀ and $\frac{2}{3}$ LD₅₀ showed a significant reduction in seminiferous epithelium height

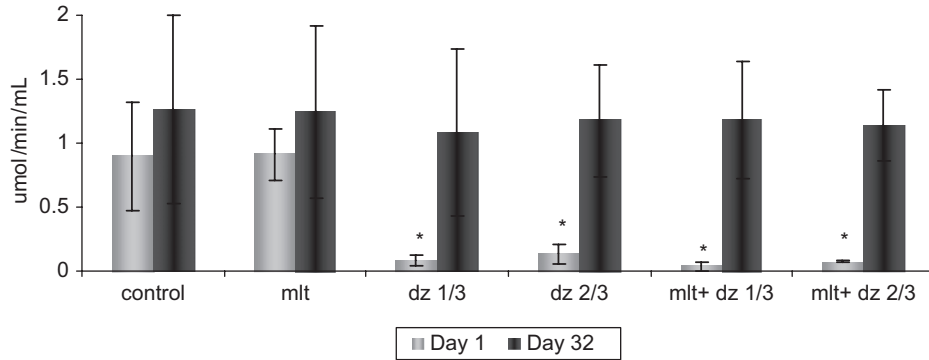


Fig. 1. Concentration of plasmatic acetylcholinesterase on days 1 and 32 pi, determined by Ellman's method. Values are expressed as mean \pm standard deviation (SD). * $p < 0.05$ vs. control; mlt = melatonin, dz = diazinon, $\frac{1}{3}$ = $\frac{1}{3}$ LD₅₀, $\frac{2}{3}$ = $\frac{2}{3}$ LD₅₀.

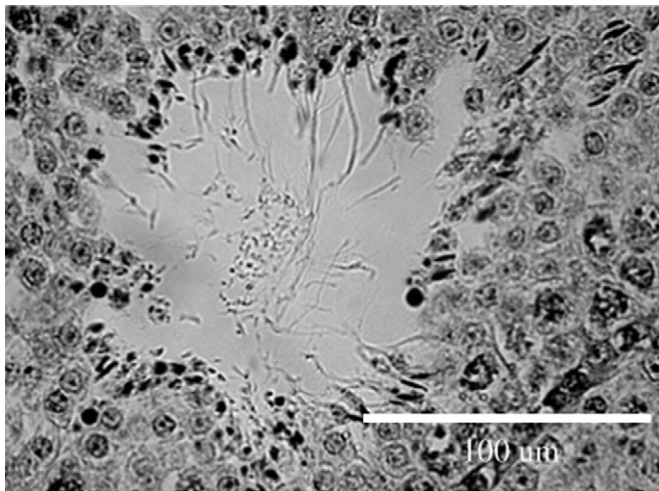


Fig. 2. Seminiferous tubule with reduced epithelial height on day 1 pi. Treatment = dz $\frac{2}{3}$ LD₅₀.

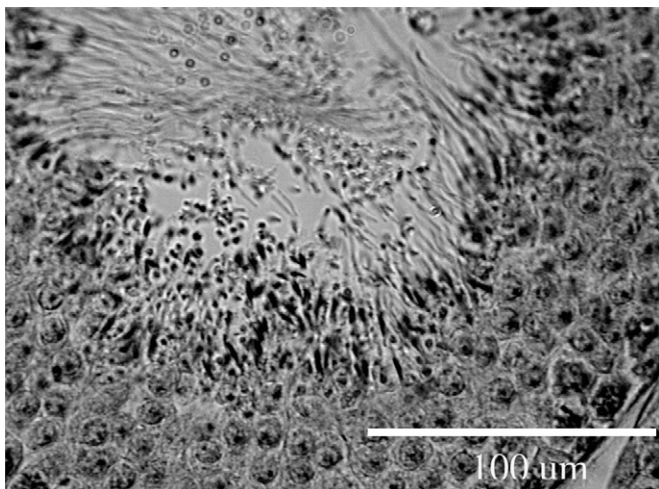


Fig. 3. Normal seminiferous tubule. Control group, day 1 pi.

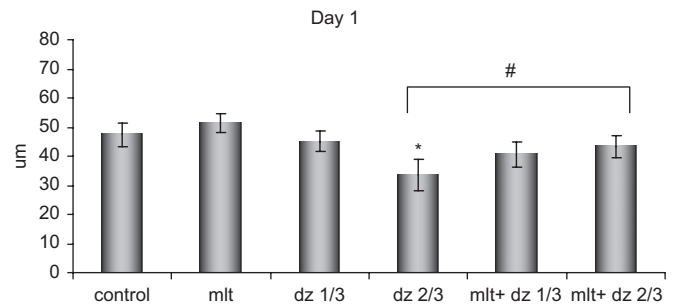


Fig. 4. Height of the seminiferous epithelium on day 1 pi. Values are expressed as mean \pm standard deviation (SD). * $p < 0.05$, dz $\frac{2}{3}$ vs. control; # $p < 0.05$ dz $\frac{2}{3}$ vs. mlt+dz $\frac{2}{3}$. dz = diazinon, mlt = melatonin. $\frac{1}{3}$ = $\frac{1}{3}$ LD₅₀, $\frac{2}{3}$ = $\frac{2}{3}$ LD₅₀.

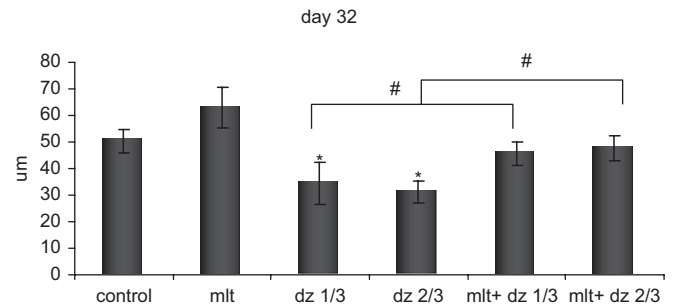


Fig. 5. Height of the seminiferous epithelium on day 32 pi. Values are expressed as mean \pm standard deviation (SD). * $p < 0.05$, dz $\frac{1}{3}$ and dz $\frac{2}{3}$ vs. control; # $p < 0.05$, dz $\frac{1}{3}$ vs. mlt+dz $\frac{1}{3}$ and dz $\frac{2}{3}$ vs. mlt+dz $\frac{2}{3}$. dz = diazinon, mlt = melatonin. $\frac{1}{3}$ = $\frac{1}{3}$ LD₅₀, $\frac{2}{3}$ = $\frac{2}{3}$ LD₅₀.

these groups of animals were significantly higher than those observed in the groups treated with dz $\frac{1}{3}$ and dz $\frac{2}{3}$ LD₅₀ (Fig. 5).

3.3. Diazinon and melatonin effect on testis interstitial area

At day 1 pi, no significant differences were detected in testis interstitial tissue, neither in groups treated with dz $\frac{1}{3}$ LD₅₀ and dz $\frac{2}{3}$ LD₅₀ (11 \pm 2% and 10 \pm 1%, respectively) nor in groups previously treated with mlt+dz $\frac{1}{3}$ LD₅₀ and mlt+dz $\frac{2}{3}$ LD₅₀ (11 \pm 2% and 8 \pm 1%, respectively) with respect to control (9 \pm 1%). On day 32 pi, we obtained the same results as on day 1 pi; no significant differences took place in testis interstitial tissue, neither in groups treated with dz $\frac{1}{3}$ LD₅₀ and dz $\frac{2}{3}$ LD₅₀ (12 \pm 2% and 10 \pm 1%, respectively) nor in groups treated before with mlt+dz $\frac{1}{3}$ LD₅₀ and mlt+dz $\frac{2}{3}$ LD₅₀ (10 \pm 2% for both groups) with respect to control (9 \pm 1%).

(34 \pm 8 and 31 \pm 4 μ m, respectively) in comparison with control (50 \pm 4 μ m). Unlike observed results in the groups treated with mlt+dz $\frac{1}{3}$ and mlt+dz $\frac{2}{3}$ LD₅₀, no changes were evident in the seminiferous epithelial height with respect to control (46 \pm 4 and 48 \pm 5 μ m, respectively). Nonetheless, epithelial heights in

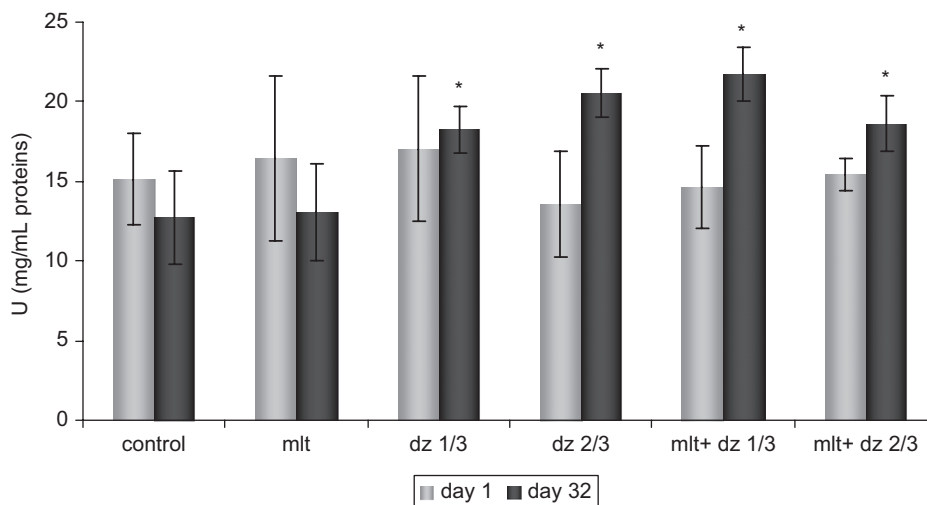


Fig. 6. Testicular SOD activity at days 1 and 32 pi. Values are expressed as mean \pm standard deviation (SD). * $p < 0.05$. dz = diazinon, mlt = melatonin. $\frac{1}{3} = \frac{1}{3} LD_{50}$, $\frac{2}{3} = \frac{2}{3} LD_{50}$.

3.4. Diazinon and melatonin effect on the activity of superoxide dismutase (SOD) in testis at day 1 pi and day 32 pi

The treatments with mlt, dz or the combination of both produced no significant variations in SOD activity levels when compared with control (15.1 ± 2.8 U mg/mL proteins) at day 1 pi. Nevertheless, at day 32 pi, a significant increase in SOD activity was observed in all groups treated with diazinon, independently of the presence or absence of previous melatonin ($p < 0.05$, Fig. 6).

3.5. Diazinon and melatonin effect on testosterone plasmatic levels

At day 1 pi, treatments with melatonin, diazinon or the combination of both did not produce any significant variation in plasmatic testosterone levels compared to control (114 ± 23 ng/dL). The same occurred with the different treatment groups on day 32 pi when compared to control.

4. Discussion

In our study, the first parameter determined was the degree of animal poisoning by measuring acetylcholinesterase activity using the Ellman's method; our results showed that all groups treated with diazinon had a significant reduction in plasmatic acetylcholinesterase activity levels. However, these decreases appear only on day 1 pi, and pretreatment with melatonin did not prevent it on any evaluated group or post-treatment time. As previously mentioned, the pesticide-induced inhibitory mechanism of acetylcholinesterase activity is not via the formation of free radicals but rather through direct enzyme inhibition. On day 32 pi, the lack of differences in cholinesterase activity observed between the treated and control groups might be due to the presence of hepatic esterases with detoxification functions, as proposed by Sams and Mason (1999).

The decrease in the height of the seminiferous epithelium without changes in tubule diameter in the group treated with diazinon (on both days 1 and 32 pi) suggests that the pesticide specifically affects the proliferation of spermatogonia—the spermatogenic cell type in active mitotic process. This would explain the fact that 32 days after treatment a decrease in the seminiferous epithelium height—possibly due to a reduction in the number of all cell types—was found. No changes were observed on the interstitial area of the testis, on the seminiferous tubule diameter measurements or in plasma testosterone on days

1 or 32 pi. However, on day 1 pi, we found a decrease of the height of the epithelium only in the group treated with $\frac{2}{3}$ of LD_{50} for diazinon, a phenomenon possibly more likely associated to an augment in apoptotic events than to a reduction in proliferative processes. This suggests that an acute exposure to diazinon induces apoptosis in the male germ cells, which leads to a lower height in the seminiferous epithelium as observed on day 32 after treatment, even with a concentration of $\frac{1}{3}$ of LD_{50} of diazinon (a phenomenon that was not registered the day after the pesticide administration). On day 32 pi, the decreased seminiferous epithelium could be due to a combination of reduced proliferation and augmented apoptotic events. Between germ cells, spermatogonia are most sensitive to pro-oxidant agents when compared to spermatocytes and spermatids (Bjørge et al., 1996). The reason for this is that they reside and develop below the junctional complex between adjacent Sertoli cells (Billig et al., 1996).

Our study determined that, in concurrent treatment with diazinon, melatonin (a molecule with powerful antioxidant properties) prevents this damage, suggesting that the toxic effect of diazinon on the testicle is mediated mainly by ROS generation. ROS are thought to be neutralized with melatonin, since damages are not observed in diazinon-treated individuals that previously received melatonin. Furthermore, as it was reported in a previous study with a rat varicocele model, the increase in the height of the seminiferous epithelium at day 32 after treatment with melatonin could be only due to the fact that melatonin increases antioxidant enzymes such as GSH-Px and catalase, which avoid ROS increase and hence the activation of the pro-apoptotic protein Bax, thus reducing apoptosis of spermatogonia, spermatocytes and spermatid cells (Onur et al., 2004). This is of great importance for the seminiferous epithelium, as it has been shown that this tissue has an elevated apoptosis rate (Kirsi et al., 2004).

Regarding SOD, our findings show that diazinon produces an increase in the testicular levels of this enzyme at day 32 pi. In a previous study (Onur et al., 2004), melatonin was injected in rats every day during 30 days until sacrifice; this chronic treatment explains their findings of augmented SOD levels. In our experiments, on the other hand, melatonin did not increase SOD levels, neither 1 nor 32 days pi, since it was injected on a single dose only. However, although melatonin did not significantly increase the levels of SOD on the studied days, we cannot discard that melatonin might increase the levels of SOD if administered during the whole experimental period, as found by Onur et al. (2004). Other studies demonstrated that pesticides such as pyrethroids derivatives (Kale et al., 1999) or the organophosphate malathion

(Muñoz-Hoyos et al., 1998) produce an increase in SOD activity in the liver. This suggests an activation of compensatory mechanisms as a response to the pesticide, a hypothesis which coincides with findings reported by Akhgari et al. (2003), and that might explain the increase of SOD activity 32 days after diazinon injection.

Almost all circulating testosterone in male blood plasma is secreted by Leydig cells located in the testicular interstice. Therefore, a reduction in plasma testosterone levels indicates an alteration in these cells (Bustos-Obregon and Gonzales-Hormazabal, 2003), which are of great importance for the progression of the spermatogenic process (Bustos-Obregon and Croxatto, 2003). Frungieri et al. (2005) demonstrated that melatonin binds to mel 1A receptors in Leydig cell *in vitro*, and that this binding blocks both the stimulus of hCG and the production of AMPc necessary for the synthesis of enzymes which participate in the transformation of cholesterol into testosterone (e.g. 17- and 3- β -hydroxysteroid dehydrogenase) and which also reduce the expression of the StAR protein. Nevertheless, in this study we did not find differences in the levels of plasmatic testosterone between the groups treated with diazinon, melatonin or the combination of both in any of the exposure periods studied. This does not rule out a direct, local effect of the assayed compounds on the local synthesis or metabolism of testosterone.

In conclusion, melatonin prevents the testicular damage caused by an acute exposure to diazinon, and the adverse long-term effects of the pesticide on the seminiferous epithelium. Diazinon can indirectly stimulate a local increase in SOD activity in the testicular tissue as a result of a cell self-protection mechanism.

Acknowledgments

The authors would like to express their gratitude for the invaluable assistance of Dr. Ramon Rodrigo (School of Medicine) for the SOD technique, to Dr. Carmen Romero and Dr. Jaime Espinosa for Testosterone RIA assays at the University of Chile Clinical Hospital, to Dr. Carlos Yañez (Chilean Public Health Institute) for acetylcholinesterase assays.

This work was financed by the School of Medicine, University of Chile. Protocols for animal experiments were approved by the Institutional Committee for Animal Bioethics, and agreed with the international regulations on the matter. Thanks are due to Arama Lab (Santiago, Chile) for kindly providing melatonin.

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