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Melatonin prevents damage elicited by the organophosphorous pesticide diazinon on mouse sperm DNA ☆

L. Sarabia *, I. Maurer, E. Bustos-Obregón

Program of Anatomy and Developmental Biology, School of Medicine, University of Chile, Independencia 1027, Santiago, Metropolitana, Chile

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ABSTRACT

Toxic effects of pesticides are commonly associated with DNA damage. To evaluate the effect of the organophosphate diazinon on sperm DNA and to test whether melatonin could prevent this damage, male mice were intraperitoneally treated with melatonin, diazinon (1/3 or 2/3 LD_{50}) or both; cauda epididymal spermatozoa were obtained on days 1 and 32 postinjection and tested for DNA alterations. On day 1, sperm from diazinon-treated mice showed augmented DNA breakages and reduced chromatin packaging, whilst DNA damage increased only in the diazinon 2/3 LD_{50} group. Micronucleus test of bone marrow cells demonstrated somatic cell chromosomal damage in both diazinon-treated groups. Pretreatment with melatonin before diazinon acute administration improved all parameters studied on day 1 pi. The organophosphorous pesticide diazinon is a dose-dependent testicular toxicant that alters the sperm DNA structure; melatonin is able to prevent this damage.

1. Introduction

Among the chemical compounds most commonly used for the control of agricultural plagues, organophosphorous pesticides (OP) like diazinon posses a great potential of acute toxicity for exposed animals and men. Diazinon (O–O diethyl O-2-isopropyl-6-methylpyrimidine-4-yl-phosphorothioate; ATSDR, 1997) is a synthetic chemical substance with broad-spectrum insecticide activity (WHO, 1998). Organophosphates have demonstrated alkylating and clastogenic properties; thus, they are capable of inducing changes in the genetic material, and they are potentially mutagenic and/or carcinogenic (Wild, 1995).

It has been demonstrated that pesticides can interfere with reproduction. The effects of pesticides on male fertility began to generate interest after Whorton et al. (1977) who associated the exposure to the nematocide dibromochloropropane (DCBP) with male infertility on human beings. Other studies have also correlated occupational exposure to pesticides with infertility in farmers (Strohmer et al., 1993). It has been observed that the steps of the spermatogenic process—from the early phase of spermatogonial proliferation, the formation of spermatocytes, their conversion into spermatids and their subsequent differentiation—constitute potentially susceptible events for the deleterious effects of environmental toxicants or DNA-damaging agents (such as drugs or radiation) that are capable of directly or indirectly affecting the testis. These agents can produce cellular or genomic damage in the germinal epithelium and generate carcinomas and/or inheritable defects (Bjørge et al., 1996).

A direct testicular toxic effect has been verified for parathion (a powerful OP), either pure or as a commercial formulation, both in the immature (Sobarzo and Bustos-Obregón, 2000) and in the adult mouse (Bustos-Obregón et al., 1998), with concomitant increment of the rate of teratozoospermia and alteration in chromatin quality of epididymal spermatozoa, and increase of apoptosis in the germ cell line (Sobarzo and Bustos-Obregón, 2000; Bustos-Obregón et al., 2001). In mouse and human seminiferous tubules cultured *in vitro*, parathion and its metabolite paraoxon inhibit spermatogonial proliferation (Rodríguez and Bustos-Obregón, 1998). Phosphine, which is broadly used as a domestic insecticide and as a rodenticide, turns into its gaseous form after application and increases the appearance of chromosomal aberrations in peripheral lymphocytes (Garry et al., 1989), thus being considered as a genotoxic substance capable of generating micronuclei in bone marrow and spleen lymphocytes (Barbosa et al., 1994). These effects are partially due to the overproduction of free oxygen radicals (Melchiorri et al., 1995). The generation of free radicals causes multiple ruptures in the

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* Corresponding author. Fax: +56 2 7373158.
E-mail address: lsarabia@med.uchile.cl (L. Sarabia).

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DNA molecule and deoxyribose degradation, both likely consequences of the formation of the hydroxyl radical (OH). It damages DNA seriously, giving origin to the beginning of mutagenic and carcinogenic processes (Meneghini and Martins, 1993). In fact, diazinon-induced oxidative stress and ROS generation have been observed after acute toxicity in an *in vivo* murine model (Teimouri et al., 2006; Giordano et al., 2007; Sutcu et al., 2007).

The well-demonstrated antioxidant properties of melatonin make it an efficient molecule for the protection of cell macromolecules from oxidative damage. For instance, melatonin 2 mM reduces the damage induced by ionizing radiations on DNA by 70%, in comparison to dimethylsulfoxide (DMSO, another well-known radioprotective agent), of which a concentration of 1 M is required to produce a similar protection level (Reiter et al., 1995). Although the protective mechanism of melatonin has not been completely clarified in most of the cases (according to the exhaustive review of Reiter, 2002), it is of our interest to investigate whether melatonin can prevent the cytotoxic and/or genotoxic effects of OP diazinon on both, mouse somatic (evaluated by the micronucleus test on bone marrow cells) and germ cells (cauda epididymal sperm), under conditions of acute toxicity. Additionally, it is important to elucidate whether a previous administration of protective melatonin could prevent the testicular toxic effects caused by a subsequent exposure to a single OP administration.

2. Materials and methods

2.1. Chemicals

Melatonin 99.9% was kindly provided by Arama Laboratories (Santiago, Chile) and a commercial formulation of Diazinon (60% w/v active ingredient) was purchased from Eximerk Laboratory (Santiago, Chile).

2.2. Animals

Seventy-two (72) adult CF-1 mice of 12 weeks of age were maintained in our lab's animal housing facilities under controlled light conditions (12 h light:12 h darkness) and temperature between 18 and 20 °C. Animals were provided with pelleted food and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined by the Bioethics Committee from the University of Chile Medical School. Mice were separated in 12 cages of 6 individuals each. All mice were injected with 200 μL of solution in a single injection. Mice from the control group (Group 1) were injected intraperitoneally (ip) with vehicle (ethanol 3% in NaCl 0.9%). The experimental groups were ip injected with diazinon (dz) properly diluted in vehicle to reach 1/3 of the lethal dose 50 (1/3 LD_{50}, Group 2) or 2/3 LD_{50} (Group 3), or with melatonin (mlt) alone (Group 4), mlt+dz 1/3 LD_{50} (Group 5) or mlt+dz 2/3 LD_{50} (Group 6). All groups were sacrificed on days 1 and 32 postinjection (pi). The groups injected with melatonin plus diazinon received melatonin 30 min before diazinon application. The dose of melatonin was 10 mg/kg of body weight (b.w.); for diazinon, the LD_{50} was previously determined in 65 mg/kg b.w. via intraperitoneal injection (data not shown).

2.3. Extraction of spermatogenic cells

Once the testes were extracted, they were freed from the tunica and cut with a scalpel blade until obtaining pieces of approximately 1 mm^3 or less. The macerated tissue was received in an Eppendorf tube containing 1.5 mL of a trypsin solution (8 mg/mL trypsin in PBS/BSA 0.1%) for enzymatic digestion. After mechanical disgregation with a Pasteur pipette, a 5 min centrifugation step at 1800*g* was carried out. The pellet was resuspended in an Eppendorf tube containing 3 mg of hyaluronidase and 0.9 mg of colagenase for a second digestion for 30 min at 34 °C in a thermoregulated bath with a 90 cycles/min agitation. The pellet was resuspended to a final concentration of 2×10^6 cells/mL.

2.4. Comet assay in spermatogenic cells

Spermatogenic cells obtained in the previous step were treated according to Haines et al. (2002). Cells were visualized at 400× using an epifluorescence microscope (Carl Zeiss, Germany) with an excitement filter of 510–560 nm and a filter barrier of 590 nm. Positive comet cells had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by DNA, which contained broken strands that were drawn away during electrophoresis. Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample to determine the percentage of nuclei with fragmented DNA in the control and treated groups.

2.5. Stability of cauda epididymal sperm DNA

Spermatozoa were obtained by mechanical disgregation of cauda epididymis in PBS; they were filtered to remove debris, washed twice with PBS, boiled at 90 °C for 6 min and immediately fixed in Carnoy solution overnight. Thick smears were made and the slides were immediately read on a fluorescence Zeiss M01 microscope in epifluorescence configuration. The procedure was previously described by Tejada et al. (1984).

2.6. Sperm chromatin decondensation

Spermatozoa from cauda epididymis were obtained by mechanical disgregation of the tissue in PBS, filtered and washed twice with PBS and the Thioglycolate assay was performed. This test analyzes the state of the sperm chromatin condensation by exposing the cell to a strongly reducing substance as sodium thioglycolate (0.4 M, pH 9.0). For this test, 0.9 mL of the reducing reagent was mixed with 0.1 mL of a sperm suspension at room temperature during 10 min. A smear was made and stained with aniline blue 5% during 5 min, dried and mounted for microscope observation with high magnification. Two patterns were observed: (1) condensed sperm chromatin, with a head of habitual size; and (2) decondensed sperm chromatin, when the head swelling reaches 50% or more than the normal head volume. Spermatozoa were observed at 100× and the size of the sperm heads was measured with a 1-mm^2 reticulated ocular. Two hundred spermatozoa were evaluated per animal, according to Fornés and Bustos-Obregón (1994).

2.7. Micronucleus assay

Only on day 1 posttreatment, a femur was removed from each mouse, cleaned up, and the bone marrow was extracted with 0.3 mL of PBS. The cell extract from each mouse was centrifuged at 800*g* for 5 min with PBS, and the pellet was resuspended and spread on slides, which were then stained with Giemsa 6% in Sörensen buffer (pH 6.8) for 30 min, washed in buffer and mounted. The results of the assay were determined by the number of micronucleated cells in 300 polychromatic erythrocytes and expressed as a percentage (Ellahueñe et al., 1994).

2.8. Statistical analysis

All data were analyzed using the nonparametric Kruskal–Wallis test, followed by the post hoc test of Dunn. Results are presented as the mean ± standard deviation of the mean (SD). The p values smaller than 0.05 ($p<0.05$) were considered as statistically significant.

3. Results

3.1. Effect of diazinon and melatonin on DNA fragmentation in cells of the germinal epithelium of the testis

At day 1 pi, the group of animals treated with dz 2/3 LD_{50} showed an increase of epididymal sperm cells with fragmented DNA (34 ± 4%, Fig. 2A). No significant differences were detected between the groups treated with dz 1/3 LD_{50} (17 ± 1%), or mlt+dz 1/3 and mlt+dz 2/3 LD_{50} (16 ± 1% and 19 ± 4%, respectively) when compared to control (16 ± 2%, Fig. 1). On day 32 pi, the group of animals treated with dz 2/3 LD_{50} (18 ± 1%) showed no difference with respect to control (15 ± 2%) (Fig. 2B), suggesting a recovery in the integrity of DNA in this interval of time.

3.2. Effect of diazinon and melatonin on the compaction of sperm chromatin

On day 1 pi, diazinon reduced the percentage of sperm with compact chromatin in the group treated with dz 2/3 LD_{50} (63 ± 8%) when compared to the control (89 ± 6%), and also when compared to the mlt+dz 2/3 LD_{50} (81 ± 5%; Fig. 3). However, on

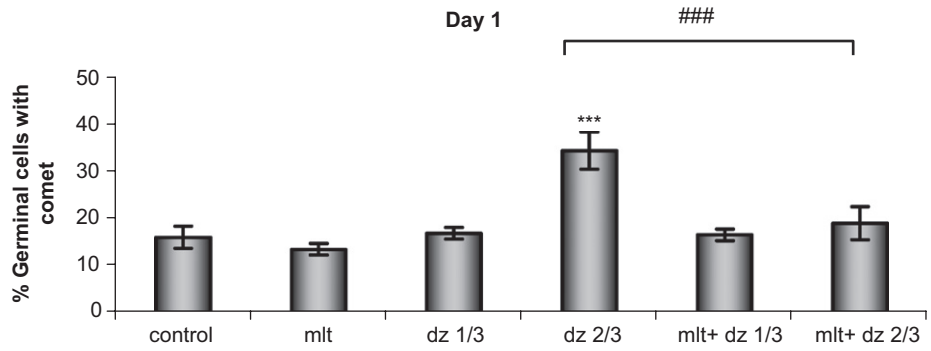

Fig. 1. Fragmentation of DNA in cells of the germinal epithelium of testis at day 1 pi determined in samples with over 68% of primary spermatocytes. The values are expressed as mean ± standard deviation (*** $p<0.001$ dz 2/3 versus control; ### $p<0.001$ dz 2/3 versus mlt+dz 2/3. dz = diazinon, mlt = melatonin. 1/3 = 1/3 of the LD_{50}, 2/3 = 2/3 of LD_{50}).

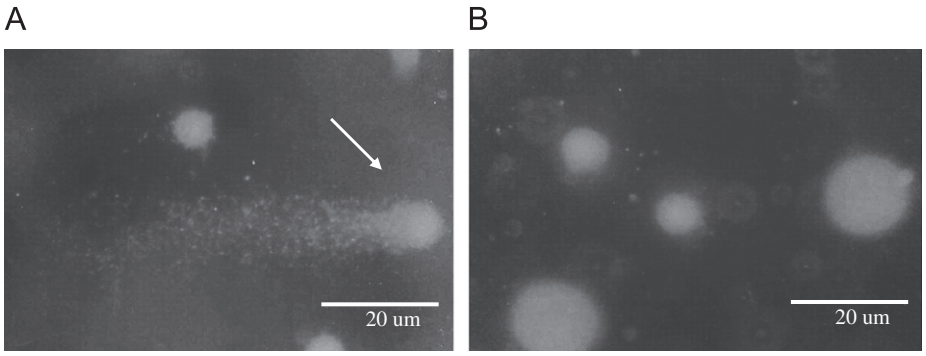

Fig. 2. (A) Primary spermatocyte, diazinon 2/3 LD_{50} at day 1 pi. The arrow indicates a nucleus with fragmented DNA. (B) normal nuclei of four primary spermatocytes with Intact DNA; diazinon 2/3 LD_{50} at day 32 pi; comet assay.

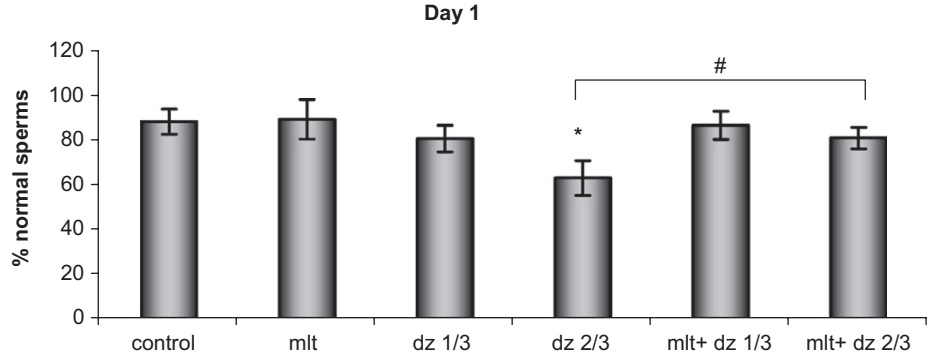

Fig. 3. State of chromatin compaction at day 1 pi. in sperm treated with sodium thioglycolate. Values are expressed as mean ± standard deviation (* $p<0.05$, dz 2/3 versus control; # $p<0.05$ dz 2/3 versus mlt+dz 2/3. dz = diazinon, mlt = melatonin. 1/3 = 1/3 of the LD_{50}, 2/3 = 2/3 of the LD_{50}).

day 32 pi, there were no differences in chromatin compaction between any groups (Fig. 4).

3.3. Effect of diazinon and melatonin on the stability of sperm DNA

On day 1 pi, diazinon induced an increase of spermatozoa with single-stranded DNA (ssDNA) in the group treated with dz 1/3 LD_{50} (24 ± 1%) as well as with dz 2/3 LD_{50} (31 ± 2%), both versus control (17 ± 2%). Nevertheless, in the group pretreated with melatonin and then with 1/3 LD_{50} of diazinon, the levels of sperm with ssDNA were kept similar to the control; but in the group that was previously treated with melatonin and then with diazinon 2/3 LD_{50}, melatonin pretreatment only diminished slightly the augment of sperms with ssDNA (Fig. 5). On day 32 pi, levels of ssDNA in sperm from both groups treated with diazinon showed no difference with respect to control.

3.4. Effect of diazinon and melatonin on the percentage of polychromatophilic erythrocytes with micronuclei

This test was applied only on day 1 posttreatment. An increment in the percentage of micronucleated cells was observed in the group treated with dz 2/3 LD_{50} (28 ± 1%) and with 1/3 LD_{50} (14 ± 1%) with respect to control (6 ± 1%) and when, respectively, compared to the groups pretreated with melatonin (9 ± 1% and 6 ± 1%, respectively; Fig. 6).

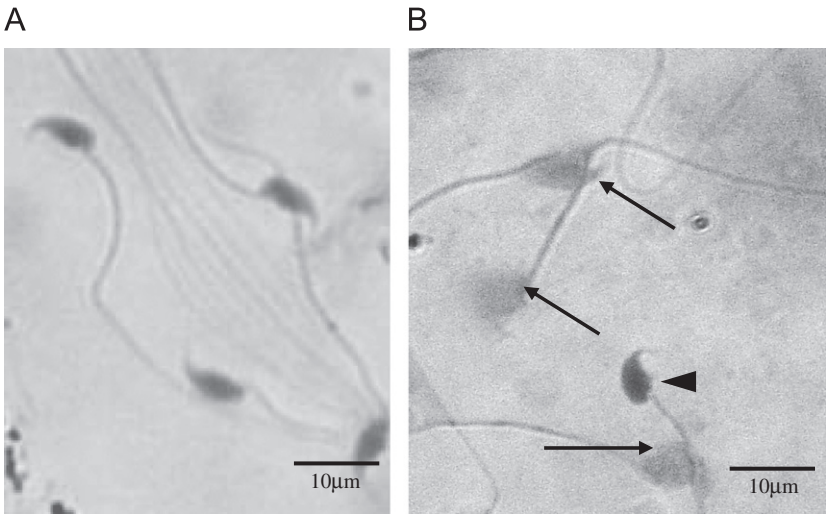

Fig. 4. (A) Sperm with normal condensed chromatin. Control at day 1 pi. (B) Arrows shows spermatozoa with decondensed chromatin and one sperm with condensed chromatin (arrowhead); diazinon 2/3 at day 1 pi.

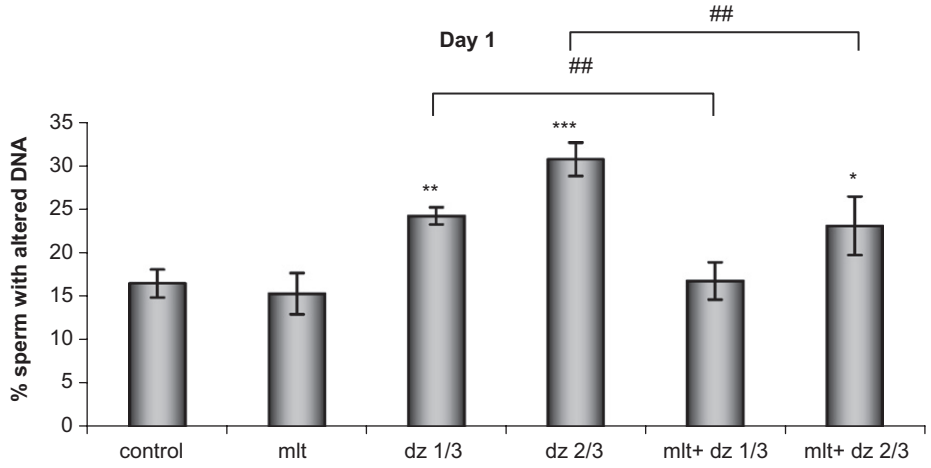

Fig. 5. Effect of diazinon and melatonin in the stability of sperm DNA at day 1 pi as determined by the acridine orange test. Values are expressed as average ± standard deviation. (*$p<0.05$ mlt+dz 2/3 versus control; **$p<0.01$ dz 1/3 versus control; ***$p<0.001$ dz 2/3 versus control; ## $p<0.01$ dz 1/3 versus mlt+dz 1/3 and dz 2/3 versus mlt+dz 2/3. dz = diazinon, mlt = melatonin. 1/3 = 1/3 of the LD $_{50}$, 2/3 = 2/3 of the LD_{50}).

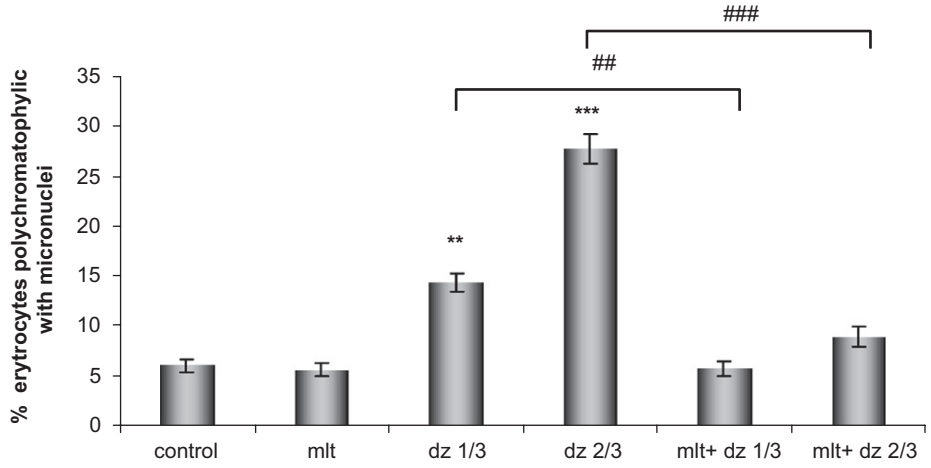

Fig. 6. Percentage of polychromatophilic erythrocytes with micronuclei determined in bone marrow smear on day 1 pi. The values are expressed as averages ± standard deviation (**$p<0.01$ dz 1/3 versus control; ***$p<0.001$ dz 2/3 versus control; ##$p<0.01$ dz 1/3 versus mlt+dz 1/3; ###$p<0.001$ dz 2/3 versus mlt+dz 2/3. mlt = melatonin, dz = diazinon. 1/3 = 1/3 of the LD_{50}, 2/3 = 2/3 of the LD_{50}).

4. Discussion

One of the mechanisms by which a toxic can alter the male reproductive function is by altering DNA or its associated proteins in the testis (Wyrobek and Bruce, 1975). According to the findings reported in the present study, the hydrogen bonds of the DNA double chain are the most sensitive structures to chromatin-damaging agents. In our results, germinal cells exhibited chromosomal fragmentation only at day 1 pi. In addition, diazinon provoked a nonlethal, cytotoxic damage on germ cells that could induce mutations in the genetic material of spermatocytes and permanent genetic changes in sperm, as observed on days 1 and 32. It has been demonstrated elsewhere that organophosphates are able to alter the homeostatic balance between cell proliferation and apoptosis (Billing et al., 1996). Regarding this feature, it has been observed that parameters for optimal semen quality have declined in humans during the last decades. Carlsen et al. (1992)—after a meta-analysis of spermograms performed between the years 1940 and 1990—associated this phenomenon with a progressive increase in exposures to chemical pollutants, with pesticides among them. On the other hand, some studies have revealed that organophosphates have alkylating and clastogenic properties (Wild, 1995). Our results demonstrate the potential of diazinon to induce fragmentation of DNA in cells of the testicular spermatogenic tissue *in vivo*. This damage becomes evident only at diazinon concentrations that reached 2/3 of the pesticide's LD_{50} on day 1 pi. However, in animals pretreated with melatonin previously to a high dose of diazinon, an inhibition in the fragmentation of sperm DNA was found in agreement with the findings reported by Tan et al. (1993). In that study, the most interesting result was that the protection of melatonin against safrole-induced DNA damage was obtained with a very low dose of melatonin.

The appearance of nuclei with broken DNA into small fragments in the control group can be explained by the fact that the spermatogenic cells have a high proliferation rate and, therefore, mechanisms of population regulation and homeostasis preservation—such as apoptosis—will normally occur (Billing et al., 1996; Bustos-Obregón et al., 2001).

In this study, we found that diazinon produced a significant decrease in the percentage of spermatozoa with compacted chromatin in the group treated with an elevated dose of the pesticide, but it was avoided with a pretreatment with melatonin. However, we did not find a significant decrease in the group treated with a lower dose of diazinon. This fact is particularly interesting, since the acridine orange test showed destabilization of DNA at both doses of diazinon and in the group pretreated with melatonin before a high dose of diazinon (2/3 of its LD_{50}). Diazinon, in a dose of 1/3 of its LD_{50}, produces openings in the double strand of spermatic DNA (as determined with the acridine orange test), but no alteration in the binding of protamines to DNA (as determined by the sodium thioglycolate test). At day 1 pi, we observed damage in sperm present in the epididymis and in testicular spermatocytes; however, on day 32 pi, the maturation of spermatocytes into epididymal spermatozoa may explain the recovery on all tested parameters we found. Moreover, Tijimes et al. (1996) determined that rat testes are capable of synthesizing melatonin due to the local presence of the enzymes necessary for the transformation of serotonin into melatonin, thus protecting the testes against free radicals.

The micronucleus test has been widely used to detect effects of genotoxicity of environmental mutagens and it has been proposed as a "screening method" to determine the clastogenic potential or aneuploidy produced by toxic substances (Mayashi et al., 1989). In our study, we found that diazinon increases the quantity of polychromatic erythrocytes with micronuclei; at the same time, we observed that in animals pretreated with melatonin prior to diazinon administration, there was no increase in micronuclei rates (Fig. 6). These results are congruent with a previous report where melatonin avoided the increment of micronuclei in polychromatic erythrocytes in peripheral blood as a consequence of the use of the OP paraquat, both 24 and 48 h postinjection (Ortiz et al., 2000). Therefore, diazinon also produces alterations of DNA in somatic cells. Tan et al. (1999) demonstrated highly elevated levels of melatonin in bone marrow; this fact is interesting for a possible major level of protection against toxic agents in this tissue.

Mammalian sperm continue to develop during their passage through the epididymis, acquiring a mature motility pattern and fertilizing ability (Hamilton, 1972). These and other changes in sperm are influenced by absorptive and secretory functions of the epididymal epithelium that permits the passage of substances of different chemical structures, such as glycerylphosphorylcholine, carnitine, steroids and sialic acid (Hamilton, 1972). The possibility that Diazinon may cross the epididymal epithelium based on its lipophilic properties and reach the stored spermatozoa would explain its damaging effects on chromatin compaction, DNA stability and sperm lipoperoxidation observed on day 1.

In conclusion, melatonin prevents the damage on DNA and the adverse effects on spermatozoa induced by diazinon *in vivo*, conserving the quality of chromatin. This action begins in the testicle, protecting the germinal epithelium from the clastogenic action of Diazinon. More studies are necessary to determine the precise molecular mechanism for melatonin protective action, which, as we hypothesize, might be based on its powerful antioxidant properties.

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