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Protective Effect of Melatonin on Damage in the Sperm Parameters of Mice Exposed to Diazinon

Efecto Protector de la Melatonina sobre el Daño en los Parámetros Espermáticos de Ratones Expuestos a Diazinón

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SUMMARY: Since normal sperm parameters can be altered by organophosphorous pesticides, this study intended to determine if melatonin is able to prevent the damage on sperm quality after an acute exposure to diazinon. Adult male mice were injected intraperitoneally with melatonin, diazinon (1/3 or 2/3 LD50) or both, and sperm parameters were evaluated on days 1 or 32 post injection. Groups treated with diazinon showed elevated lipid peroxidation levels on day 1 post treatment, while groups pretreated with melatonin before diazinon showed no difference compared to control. Sperm count showed a significant decrease in both diazinon-treated groups only on day 32 post injection; no differences were observed in groups pretreated with melatonin prior to diazinon compared to control. The percentage of abnormal sperm morphology increased in the diazinon-treated groups only on day 32 postinjection. The administration of melatonin prior to exposure to diazinon prevents the alteration of sperm parameters commonly caused by organophosphates, possibly due to its antioxidant properties.

KEY WORDS: Melatonin; Organophosphates; Antioxidant; Mouse sperm; Sperm morphology.

INTRODUCTION

Organophosphorous pesticides like diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidinyl-4-g-1-phosphorothioate) are widely used for the control of agricultural plagues. Several other organophosphates, such as parathion or malathion, have shown to provoke deleterious effects on mammalian reproduction, as such the increment in teratozoospermia levels, the alteration of chromatin quality in epididymal spermatozoa, the severe decrease in sperm count and the increase of apoptosis levels in the germinal epithelium (Bustos-Obregón *et al*., 2001; Martínez-Haro *et al.*, 2008).

It has been shown that the levels of lipoperoxidation are elevated in rat heart tissue poisoned with the pesticide phosphine, and that this compound and its derivatives can produce an increment in the levels of lipid peroxidation in the rat brain, lung and liver (Hsu *et al*., 2002; El-Shenawy *et al.*, 2010). Additionally, direct sperm damage has been verified in Eisenia foetida (earth red worm) when exposed to organophosphates (Espinoza-Navarro & Bustos-Obregón, 2005); in those experiments, when melatonin was added simultaneously to the terrarium, the sperm damage was totally prevented for low pesticide concentrations or severely diminished for high concentrations of diazinon (Bustos-Obregón *et al*., 2005).

Melatonin, a hormone secreted mainly by the pineal gland, exhibits some protective functions for cells against noxious agents, acts like a powerful antioxidant and scavenger of free radicals, and counteracts the generation of free radicals by inhibiting the activity of the nitric oxide synthase, NOS (Reiter *et al*., 2001). Melatonin shows a similar activity to purify peroxide radicals (ROO-), a consequence of lipid peroxidation, thus being a more powerful antioxidant than vitamin E (Hardeland *et al*., 2011; Yassa *et al.*, 2011). Therefore, we hypothesize that melatonin could be highly specific against lipid peroxidation for several reasons: it is highly lipophilic, it is usually found in high concentrations in cell membranes and -as vitamin C and E- it reduces the oxidation of lipids due to its purifying activity

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of the OH- radical (Ahmed *et al*., 2011). Although the protective mechanism of melatonin has not been completely clarified in most of the cases it is of great interest to investigate if melatonin could prevent the cytotoxic effects of diazinon on mouse epididymal sperm parameters of good quality under experimental conditions. Our objective is to verify if this action would also occur in *in vivo* mammals after an acute exposure to the organophosphate.

MATERIAL AND METHOD

Chemicals. Melatonin (99,9% purity) was kindly supplied by Arama Lab (Santiago, Chile) and a commercial formulation of diazinon (Diazyl®, 60% active ingredient) was obtained from Eximerk Laboratories (Santiago, Chile).

Animals. Seventy two adult CF1 mice of 12 weeks of age were used for this study and maintained in controlled light conditions (12:12 hours light:dark) and temperature between 18º and 20º C. Animals were provided with food and water *ad libitum*. All animal studies were conducted in accordance with the principles and procedures outlined by the Bioethics Committee of the School of Medicine, Universidad de Chile. Male mice were separated in 12 cages of 6 individuals each and were injected with 200 uL of solution. Mice from the control group (Group 1) were injected intraperitoneally (ip) with vehicle (ethanol 3% in NaCl 0,9%). The experimental groups were injected with diazinon (dz) diluted in vehicle in the following doses: 1/3 of the LD50 (Group 2), 2/3 of the LD50 (Group 3), with melatonin (mlt) (Group 4), mlt–dz 1/3 of the LD50 (Group 5), and mlt-dz 2/3 of the LD50 (Group 6). All groups were evaluated on days 1 and 32 post injection (pi). The groups injected with both melatonin and diazinon were injected with melatonin 30 minutes before 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 via intraperitoneal injection in our laboratory (Mueller & Solecki, 2006; Sarabia *et al.*, 2009).

Sperm counts. Both cauda epididymides from each animal were weighed and macerated in 5 mL of phosphate buffer saline (PBS, 0,1 M, pH 7.2) in Petri dishes using a scalpel; homogenates were kept at 4º C for 24 hours, and then filtered through double gauze. Sperm heads were counted per cauda epididymides in a Neubauer's hemocitometer (Vigil & Bustos-Obregón, 1984).

Sperm morphology . The homogenate previously described was washed two times with PBS. The resulting pellet was resuspended in 100 mL of this buffer, and smears were prepared and fixated in 70º ethanol for one minute and stained with hematoxylin and eosin. Then, 300 spermatozoa were inspected under the microscope with a 100X objective (Vigil & Bustos-Obregón, 1984).

Analysis of lipid peroxidation. Lipid peroxidation levels were measured by the thiobarbituric acid (TBARS) test, promoted by ferrous and ascorbate ions. TBARS was determined by a double heating method. For this assay, 1 mL of sperm suspension containing 5 x 10^6 spermatozoa/ mL, previously washed in PBS, was incubated at 37º C for 1 hour in presence of 50 mL of 1mM ferrous sulphate and 25 mL of sodium ascorbate (Kodama *et al*., 1996). The reaction was stopped with 31.2 mL of trichloroacetic acid (TCA, 100%) added to each tube, and then cooled in ice-cold water for 20 min and centrifugated at 2,000 xg for 10 minutes. The supernatant is again centrifugated and then 700 mL of the supernatant was added to 1300 mL of 6.7 g/L solution of TBA in a test tube and maintained in a boiling water bath for 20 min. Tubes were cooled in ice-cold water for 5 minutes and their absorbances were measured using a Shimadzu UV 120-02 spectrophotometer (Kyoto, Japan) at 535 nm of wavelength. Lipid peroxidation was expressed as nanomoles of thiobarbituric acid reactants (TBARS) generated by 10^6 spermatozoa after 1 h of incubation.

Statistical analysis. Normality test was performed according to the Grubbs test with 95% confidence, with a significant adjustment. Analysis of variance was applied between treatments ($p \leq 0.05$) by separating the means for post-hoc test "least significant difference" (LSD). Data analysis was performed using statistical software Statgraphic Plus 5.1 for PC.

RESULTS

Sperm count (Table I).

When evaluated on day 1 after exposure, the different treatments with diazinon and/or melatonin did not produce differences in the sperm concentration with respect to control (0.7 ± 0.1 x 10^6 sperms/mg). On the other hand, on day 32 postinjection the treatment with melatonin alone produced a significant increase on sperm concentration (1.02 ± 0.09 x 10^6 sperms/mg). The groups treated with diazinon 1/3 of the LD50 (0.55 ± 0.08 x 10^6 sperms/mg) and 2/3 of the LD50 (0.41 ± 0.04 x 10^6 sperms/mg) show a significant decrease in the count with respect to control (0.74 ± 0.09 x 10^6 sperms/ mg). The group treated with diazinon 1/3 and 2/3 LD50 do

Table I. Sperm count per mg of tissue from the epididymis. Counts at 1 and 32 day post treatment. The significance analysis was performed in groups and between groups ($p \leq 0.05$). 1/3 and 2/3 = 1/3 LD50 of diazinon and 2/3 LD50 of diazinon.

Treatment	Sperm counts/ mg of epididyme			
	1 Day		32 day	
	Mean	Significance	Mean	Significance
Control	0.727	de	0.74	de
Melatonin (Mlt)	0.86	ef	1.02	f
Diazinon 1/3	0.729	de	0.55	abc
Diazinon 2/3	0.67	bcd	0.4	a
Mlt+ diazinon 1/3	0.76	de	0.7	cde
Mlt+ diazinon 2/3	0.81	de	0.51	ab

Significance level $p \leq 0.05$.

not have a significant decrease in the sperm count with respect to the melatonin-diazinon 1/3 and 2/3 LD50 groups. However when comparing treatment between moments in time (1 day and 32 days) shows a significant decrease in sperm at day 32 in groups of diazinon at doses of 1/3 and 2/3 of the LD50. In the group of melatonin-diazinon 1/3 no significant differences is observed, however in the group of melatonin-diazinon at doses of 2/3 of the LD50, if there is a significant decrease in sperm count at 32 day ($0.81 \pm 0.2 \times 10^6$ sperms/mg and $0.51 \pm 0.06 \times 10^6$ sperms/mg respectively).

Sperm morphology (Tables II, III)

Table II shows that at day 1 post treatment the groups of diazinon 2/3 alone and group melatonin + diazinon 2/3, have a significant decrease in the percentage of normal sperm (70 ± 3 and 70 ± 2 respectively), when compared to control (78 ± 4 %). Day 32 post treatment shows a significant decrease in the groups treated with diazinon alone at doses of 1/3 and 2/3 (65 ± 4 and 63 ± 3 respectively). The column of differentials shows that in the groups treated with melatonin + 1/3 of diazinon and melatonin + 2/3 of dizinon expressed a significant increase at day 32, with similar values at the control group (76 ± 3).

Table III shows the percentages of teratozoospermia in groups at 32 days post treatment. All of them observed that treatment with diazinon alone (1/3 and 2/3) increase significantly the percentages of abnormalities in head and tail (20 ± 4 % and 12 ± 2 %) with respect to control (13.4 ± 3.6 % and 7.8 ± 1 %, respectively). Also seen in the column of abnormalities that melatonin alone or in combination with diazinon, prevents the increase of teratozoopermia, whose values are very similar to the control group (24.2 ± 4.1 %). Similar behavior is observed in the columns of abnormalities of head, middle segment and tail of sperm.

Sperm lipoperoxidation (Table IV),

Table II. Normal sperm morphology was expressed as mean ± standard deviation. The significance was analyzed at each period of treatment and at day 32 the differential between them was calculated. 1/3 and 2/3 = 1/3 LD50 of diazinon and 2/3 LD50 of diazinon.

Treatment	Normal sperm morphology in percentage (mean ± SD)		
	1 Day	32 day	Differential (1-32 day)
	Mean ± SD	Mean ± SD	Mean
Control	78 ± 4	76 ± 3	+2
Melatonin (Mlt)	82 ± 6	78 ± 4	+4
Diazinon 1/3	76 ± 5 *	65 ± 4 *	-9*
Diazinon 2/3	70 ± 3	63 ± 3 *	-7*
Mlt+ diazinon 1/3	73 ± 3	80 ± 2	+7*
Mlt+ diazinon 2/3	70 ± 2 *	78 ± 4	+8*

Significance level*: $p \leq 0.05$.

Table III. Shows the percentages of teratozoospermia with abnormalities in head, middle segment and tail of the sperm collected from the epididymis. Values are expressed as mean ± standard deviation. 1/3 and 2/3 = 1/3 LD50 of diazinon and 2/3 LD50 of diazinon.

32 day post treatment	Percentage of teratozoospermia (mean ± SD)			
	Total	Head	Mid Seg (1)	Tail
Control	24.2 ± 4.1	13.4 ± 3.6	3 ± 1.2	7.8 ± 1.2
Melatonin (Mlt)	21.6 ± 5.1	12.3 ± 4.5	4.2 ± 1.7	5.1 ± 1.5
Diazinon 1/3	34.9 ± 3.1*	19 ± 2.4 *	5.8 ± 1 *	10.1 ± 1.2 *
Diazinon 2/3	37.5 ± 6.7 *	20.4 ± 3.4 *	5.1 ± 0.8 *	12 ± 2.4 *
Mlt+ diazinon 1/3	20.2 ± 5.8	12.4 ± 3.5	2.8 ± 1.4	5 ± 2.1
Mlt+ diazinon 2/3	22.1 ± 5	13.9 ± 3.9	2.3 ± 0.9	5.9 ± 0.8

Significance level*: p≤0.05. (1): Middle segment.

At day 1 post treatment, diazinon produced a significant increase in the lipid peroxidation level in the group treated with diazinon 2/3 LD50 (11.2 ± 1.6 nmol TBARS/10^6 sperms) with respect to diazinon 1/3 LD50 (9.5 ± 1.0 nmol TBARS/10^6 sperms) compared with control (5.8 ± 0.7 nmol TBARS/10^6sperms). This increase in lipid peroxidation is also significant in the group melatonin-diazinon 1/3 LD50 (6.1 ± 1.6 nmol TBARS/10^6 sperms) and diazinon 1/3 LD50 ± melatonin (6.8 ± 1.8 nmol TBARS/10^6 sperms) (Fig. 7). On day 32 post injection, only the group treated with diazinon 2/3 (7.5 ± 1.7 nmol TBARS/10^6 sperms) shows a significant increase in lipid peroxidation levels with respect to the control group (6.1 ± 0.6 nmol nmol TBARS/10^6 sperms). A comparison between treatment at days 1 and 32, shows a significant decrease in levels of lipid peroxidation at day 32 in groups treated with diazinon 1/3 and 2/3 with respect to day 1. The same happens in the group 2/3 melatonin-diazinon (6.3 ± 0.4nmol TBARS/10^6 sperms).

Table IV. Lipid peroxidation of sperm was expressed as nanomoles of thiobarbituric acid reactants (TBARS) generated by 10^6 spermatozoa. Statistical significance was analyzed by the corresponding mean.

Treatment	Lipid peroxidation/ nmol TBARS/10^6 sperms			
	Day 1		Day 32	
	Mean	Significance	Mean	Significance
Control	5.75	ab	6.15	abc
Melatonin (Mlt)	5.5	a	5.46	a
Diazinon 1/3	9.45	f	6.33	bcde
Diazinon 2/3	11.28	g	7.55	e
Mlt+ diazinon 1/3	6.86	cde	6.3	abc
Mlt+ diazinon 2/3	6.91	de	6.13	abc

Significance level: p≤0.05.

DISCUSSION

Our results show that melatonin is able to exert an *in vivo* protective effect over the damage induced by an acute exposure to the pesticide diazinon on all the analyzed parameters of mice sperm. The alterations demonstrated in spermatozoa on day 1 post injection correspond to a direct effect of the treatment (the pesticide and/ or melatonin) on spermatozoa when they were transiting by the epididymis at the moment of administration of the compounds, whereas the effects observed on day 32 post injection correspond to spermatozoa obtained from cauda epididymis that at the time of administration of the pesticide and/or melatonin were in the stage of primary spermatocyte in the epithelium of the seminiferous tubules. Therefore, these spermatozoa correspond to germ cells that survived the toxic effects of the pesticide, which might explain some alterations on sperm parameters in the analyses performed on day 32 (Sarabia *et al.).*

In the present study, we found that on day 1 post treatment, diazinon significantly increases the levels of lipoperoxidation in spermatozoa, which might explain the decreased number of sperm cells in the experimental group treated with diazinon alone on day 32 post injection, and that this decrease is greater when higher doses of diazinon are injected. Other studies have shown that malathion (another organophosphorous pesticide) induces similar changes in the mouse testis, accompanied with epithelial vacuolization and descamation of sperm germ cells with likely

deleterious effects over Sertoli cells (Contreras *et al.*, 1999; Bustos-Obregón *et al*., 2003; Dirican & Kalender, 2011; Sirajudeen *et al.*, 2011).

We found fewer sperm in the experimental group treated with diazinon 2/3 LD50 on day 32 post injection but not in the group treated in the same way at day 1 post injection. At day 1 after treatment, the counted sperm cells correspond to spermatozoa located in the epididymis. On the other hand, in animals treated with diazinon 2/3 LD50 and melatonin, we found only a slight but significant decrease in the sperm count on day 32 post injection, suggesting that melatonin exerted a preventive effect. Besides, melatonin produced an increase in sperm concentration, possibly by diminishing the basal levels of apoptosis of the germinal epithelium via diminished ROS levels (Curtin *et al.*, 2002; Martín-Hidalgo *et al.*, 2011).

With regard to sperm morphology, several studies have demonstrated that organophosphorous pesticides such as malathion interfere in sperm differentiation producing anomalies in sperm tails in treated mice which might alter fertility significantly (Contreras *et al*.). This correlates with our results observed in sperm cells on day 32 post diazinon 2/3 LD50 injection. Likewise, it has been demonstrated that parathion produces an increase in the percentage of necrospermia and morphologic sperm abnormalities of treated mice (Bustos-Obregón *et al*., 2001). At day 32, diazinon treatment produces a significant increase in the sperm morphology alterations, both in sperm head as well as in the tail, possibly due to the fact that at the moment of administration of the pesticide the analyzed epididymal sperm were at the stage of primary spermatocytes in the seminiferous tubule (Rodríguez *et al*., 2006). The injection of diazinon 1/3 DL50 did not produce changes on the sperm morphology observable with conventional light microscopy, possibly due to the fact that the mechanisms of cell repair or apoptosis are sufficient to avoid morphologic alterations, which does not imply that a more detailed analysis might demonstrate structural alterations with this dose. In the groups co-treated with melatonin plus the pesticide, morphological anomalies were not observed with both doses of the pesticide or time intervals, corroborating the preventive role of melatonin against damage induced by the pesticide possibly via melatonin receptor– and extracellular signal–regulated kinase-mediated pathways (Espino *et al*., 2011; Martín-Hidalgo *et al*.).

Our results showed that diazinon produces lipid peroxidation in sperm cells extracted from the cauda epididymis only at day 1 after pesticide exposure and a posterior recovery on day 32. Other studies have shown that lipid peroxidation increases in cardiac tissues of rats poisoned by other phosphate compounds such as phosphine (PH3) (Hsu *et al*.), and also in humans poisoned with this compound (Chung *et al.*, 1996). PH3 increased significantly the lipid peroxidation also in brain, lung and liver compared with non treated animals. Moreover, the lipid peroxidation levels did not decrease after a treatment with antioxidants alone, such as vitamin C or b-carotene, but the increase of lipid peroxidation induced by PH3 was significantly blocked by the pre-treatment with melatonin in all these tissues (Hsu *et al*.; Yu *et al*., 2008). In our study, the pre-treatment with melatonin 30 minutes before the administration of diazinon was able to completely avoid the increase of lipid peroxidation in epidydimal sperm cells from mice treated with diazinon 1/3 LD50 1 day after treatment, while this pre-treatment diminished significantly the lipid peroxidation of sperm cells from animals treated with diazinon 2/3 LD50. Another study compared vitamin E or a-tocopherol with melatonin; the latter compound showed to be more efficient *in vivo* than the vitamin and at a lower dose (10 mg/kg of body weight) and it was twice as effective as Trolox (water soluble vitamin E), reducing and neutralizing the peroxide radicals (Pieri *et al.*, 1994; Yu *et al*.). On the other hand, the lack of a significant increase of sperm lipid peroxidation at 32 days pi suggests that a fraction of the germ cells of the seminiferous epithelium is possibly eliminated by apoptosis. The results of this study support the importance of melatonin as a protective agent on the biological processes *in vivo*, especially in reproductive and seminal patterns (Reiter *et al*., 2009; Chuffa *et al*., 2011).

In conclusion, the administration of melatonin prior to exposure to diazinon prevents the alteration of sperm parameters commonly caused by organophosphates, possibly due to its antioxidant properties. Specifically melatonin has a protective effect on sperm counts, morphology and lipid peroxidation levels. Melatonin could be used to reduce the toxic damage of diazinon.

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SARABIA, L.; ESPINOZA-NAVARRO, O.; MAURER, I.; PONCE, C. & BUSTOS-OBREGÓN, E. Efecto protector de la melatonina sobre el daño en los parámetros espermáticos de ratones expuestos a diazinón. ***Int. J. Morphol., 29(4)***:1241-1247, 2011.

RESUMEN: Debido a que los parámetros normales de los espermatozoides pueden ser alterados por algunos contaminantes como los pesticidas organofosforados, este estudio pretende determinar si melatonina es capaz de prevenir o proteger del daño en la calidad espermática, después de una exposición aguda a diazinon. Ratones machos adultos fueron inyectados via intraperitoneal con diazinon 1/3 y 2/3 de la LD50 y otro grupo tratados con melatonina + 1/3 diazinon LD50 y melatonina + 2/3 LD50. Los parámetros espermáticos fueron evaluados al día 1 y al día 32 post tratamiento. Los grupos tratados con diazinon solo o conjugado con melatonina mostraron un incremento significativo en los niveles de lipoperoxidación en el tratamiento después de un día. Al día 32 no se observan diferencias significativas con el grupo control. El recuento espermático al día 1 no presenta diferencias entre los grupos tratados y el control. Sin embargo al día 32 los grupos tratados con diazinon solo, muestran una disminución significativa, solo el grupo de melatonina +1/3 diazinon, presenta valores similares al grupo control. La morfología espermática normal presenta una disminución significativa en grupos tratados con diazinon, pero un aumento significativo al día 32 en los grupos tratados con melatonina. Los mayores porcentajes de anormalidades se presentan en la cabeza y la cola de los espermatozoides. La administración de melatonina antes de la exposición al diazinon evita las alteraciones de los parámetros espermáticos, comúnmente causada por organofosforados, posiblemente debido a sus propiedades antioxidantes.

PALABRAS CLAVE: Melatonina; Antioxidantes; Ratón; Espermatozoides.

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