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Malathion Affects Spermatogenic Proliferation in Mouse

Malathion Afecta la Proliferación Espermatogénica del Ratón

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SUMMARY: The restriction of the mechanisms of cell proliferation in murine seminiferous epithelium, in terms of induction of programmed cell death until recently has not been fully analyzed. The aim of this work was to assess the effect of Malathion (MP) on testicular morphology and function in mouse spermatogenesis. For the experiments, male albino mice of strain NMRI-IVIC, weighing between 30-40 g were used, and divided into control and experimental groups of 5 each. The animals of the experimental groups were injected with a single dose of MP: 241mg/kg weight (1/12 LD 50) resuspended in 0.9% saline, intraperitoneally. Animals were sacrificed at 8.3, 16.6 and 33.2 days post-injection (first, second and third spermatogenic cycles). Testicular samples were obtained for light microscopy (LM), transmission electron microscopy procedures, and to detect apoptosis and p53 antigen by immunohistochemical methods. Blood was collected to quantify testosterone and plasmatic cholinesterase activity. From 8.3 days, Sertoli cell vacuolization, karyolisis of pachytene spermatocytes and Leydig cells and a decreased in average of the diameter of seminiferous tubules was observed. No damage to inter-Sertoli cells junctions was detected. Percentage of seminiferous tubules showing germ cells apoptosis was increased from 8.3 days, plasmatic acetylcholinesterase activity was reduced in the group treated only 24 hours after administration of MP. Serum testosterone levels were low in treated animals at 16. 6 and 33.2 days. p53 was mostly expressed in pachytene spermatocytes from 8d. The findings of this study indicate that MP alters the testicular function affecting the DNA and interfering with spermatogenesis as well as steroidogenesis.

KEY WORDS: Malathion; Testis, mouse; Apoptosis; Morphology; p53; Proliferation.

INTRODUCTION

Despite the toxic effects of organophosphates (OP) known, there are a few studies about its influence on reproduction and the gonads are generally not included in the analysis in routine toxicology studies (Hodgson, 1987; Taylor, 1996, Bustos-Obregon *et al*., 1998; Crittenden *et al*., 1998, van den Beukel, 1998). It has been reported that pesticides can cause cytogenetic disorders in somatic and germ cells, altering the process of spermatogenesis. The oxidation of sulfhydryl groups which originate from OP are highly electrophilic and bind convalently to many biomolecules such as proteins, RNA and DNA. This alkylating effect induces mutagenesis, as suggested by disruption of sperm morphology or teratozoospermia (Wyrobeck & Bruce, 1975, Chen *et al*., 1981; Sobti *et al*., 1982; Marletta, 1983; Wyrobeck, 1983; Salvadori *et al*., 1988, Jayashree *et al*., 1994, Murray & Butler, 1994).

There is evidence, however, that the OP compounds may exert a deleterious effect on the male reproductive function by its genotoxic, genetic and epigenetic actions, as they would alter gene expression without changing the nucleotide sequence. Parathion is able to interfere with the proliferation and differentiation of immature germ cells of mice, especially in the earliest periods of spermatogenesis, reversing this effect at 28 days after a single dose administration (Bustos-Obregón & Sobarzo, 2000; Rodriguez & Bustos-Obregón, 2000). Leydig cells may exhibit morphological changes indicative of cell death such as pyknosis and possibly changes in steroidogenesis. Sertoli cells may also show interference in their metabolism, although this fact has not been fully elucidated for this OP. However, despite the controversy generated around the possible mutagenic effect of OP, evidences suggest that Parathion inhibits *in vitro* proliferation of spermatogonial cells, possibly by its alkylating effect on DNA (Bustos-Obregón *et al.*, 1998; Rodriguez & Bustos-Obregón, 2000; Bustos-Obregón & Hartley, 2009).

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A study accepted by the US-EPA, regarding the evaluation of reproductive toxicity of malathion (MP) in rats is based on the measurement of variables such as the weight of the parental organisms and that of the offspring during lactation and weaning. Although EPA believes that MP has no adverse reproductive effects in the study they did not consider other aspects such as structure and function of the gonads (US-EPA, 2000).

Administration of MP in immature rats alters spermatogenesis by a decrease in the local production of testosterone (Krause *et al.*, 1975). The restriction of the mechanisms of cell proliferation in murine seminiferous epithelium, in terms of induction of programmed cell death until recently has not been associated with MP, even at sub-lethal doses.

Therefore, this study aimed to assess the effect of MP on testicular morphology and function in a murine model, and its association with serum testosterone, plasma acetylcholinesterase inhibition, proliferation and induction of programmed cell death in the seminiferous epithelium.

MATERIAL AND METHOD

For the experiments, male albino mice of strain NMRI-IVIC, weighing between 30-40 g were used; they were fed with commercial pellets and water *ad libitum* and kept in light-dark cycles of 12:12 h, at 20 ± 2 ° C in stainless steel cages. The mice were divided into control and experimental groups of 5 each. The animals of the experimental groups were injected with a single dose of MP: 241mg/kg weight (1/12 LD50) resuspended in 0.9% saline, intraperitoneally. An equivalent volume of 0.9% saline by the same route was administered to the control group. Animals were sacrificed at 8.3, 16.6 and 33.2 days post-injection (first, second and third spermatogenic cycles). After injecting the pesticide, using diethyl-ether as anesthetic, both testes were removed for histological processing for light microscopy (LM), transmission electron microscopy (TEM) and to detect apoptosis by immunohistochemical methods. Also blood was collected by cardiac puncture to quantify serum testosterone and plasmatic cholinesterase activity.

Histochemical study. Evaluation of the stages of the seminiferous epithelium cycle (Leblond & Clermont, 1952), was done on paraffin-embedded testis sections, that were stained with PAS (Hotchkiss, 1948).

Morphometry of the seminiferous tubules. Measurement of the diameter of seminiferous tubules was performed using Image Tools software (Image Tools, 2008).

Immunohistochemistry (IHC). For the detection of apoptotic cells apoTACS TUNEL staining (R & D Systems â) was used. Briefly, the sections were incubated with biotinylated monoclonal mouse anti-BrdU, then streptavidin conjugated to peroxidase was added and finally the reaction was developed with diamino-benzidine (DAB). Finally two drops of glycerin were put on slides, and covered for observation by Olympus CH30 microscope with objectives 16X, 40X and 100X.

p53 antigen was detected in paraffin-embedded testicular sections with a Novocastra Laboratories commercial kit, following the manufacturer's instructions. Briefly, sections were washed with PBS, incubated with PBS containing horse serum 10% and subsequently with anti p53 antibody overnight. Then biotynilated IgG secondary goat anti-rabbit diluted in PBS and 5% horse serum was added. To visualize bound antibodies, sections were covered with PBS to which DAB in 0.03% H2O2 was added. Sections were counterstained with Mayer´s hematoxylin.

Electron microscopy. Testicular samples portion were fixed in 3% glutaraldehyde post-fixed in 1% osmium tetroxide, and stained with lead citrate or uranyl acetate. In addition, lanthanum nitrate was used to assess the integrity of the blood-testis barrier according to Shaklai & Tavasoli (1977) technique. The sections were observed using a transmission electron microscope Philips CM-100.

Serum testosterone. To quantify testosterone EIA method (Biomerieux ®) was used, with the Mini Vidas ® apparatus.

Plasma cholinesterase. Plasma cholinesterase was quantified by spectrophotometry using a commercial test based on the method of Rappaport (Dibutyril-cholinesterase kit, Sigma-Aldrich ®).

Statistical Analysis. The evaluation of the statistical significance of the results was performed using the Student "t" test, through SPSS version 11.0 for Windows, 2000.

RESULTS

Morphology of the testis of malathion-treated mice. With respect to the control (Fig. 1), we observed the presence of vacuoles of various sizes in the cytoplasm of Sertoli cells and germ cells desquamation. These changes were evident at 8.3 days after the toxicant was administered and were

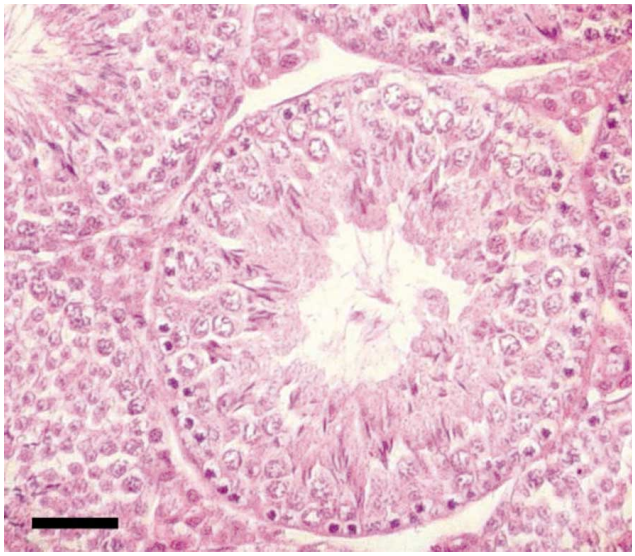

Fig 1. Testis, control group. Normal (HE, bar = 40 µm).

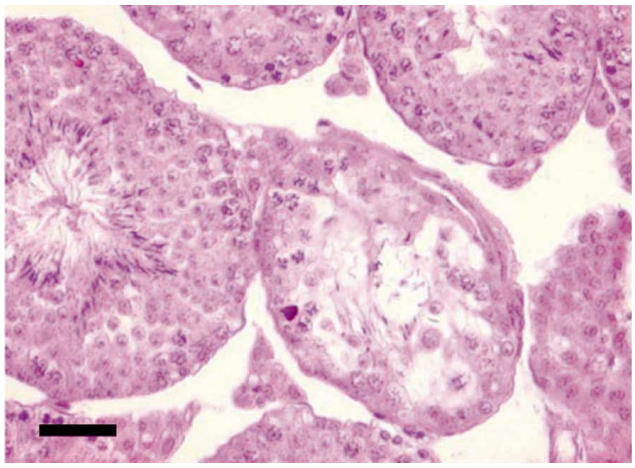

Fig 2a. Testis, malathion-treated group, 8 d. Lack of cells in seminiferous epithelium. (HE, bar= 50 µm).

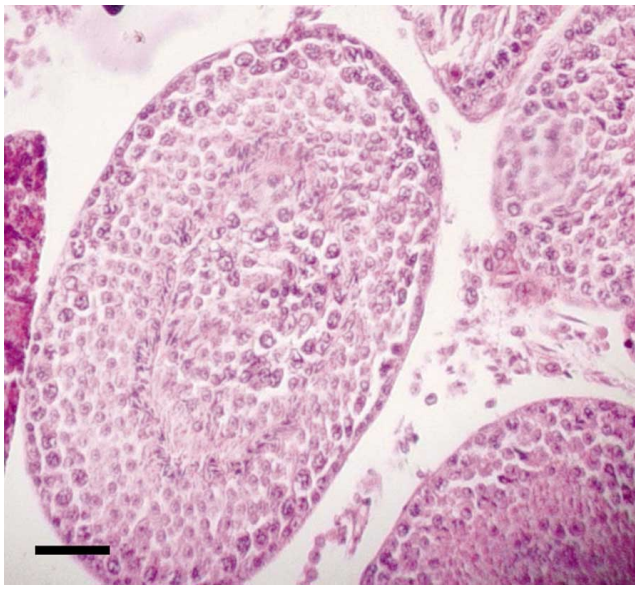

Fig 2b Testis, malathion-treated group, 16.2 d. Cell desquamation. (PAS, bar = 40µm).

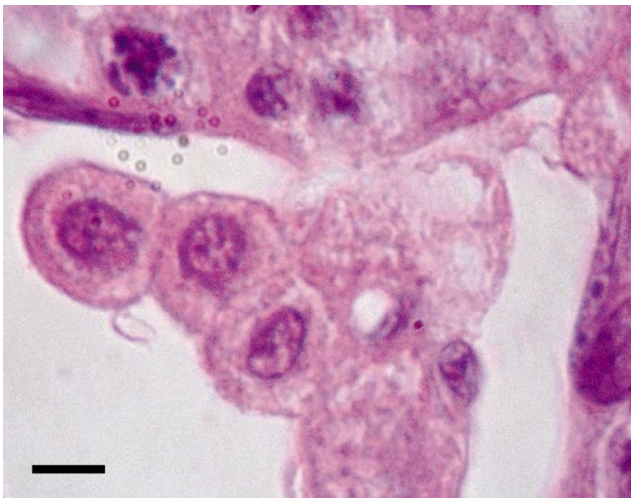

Fig 3a. Control. Normal Leydig cells (HE; bar = 15µm).

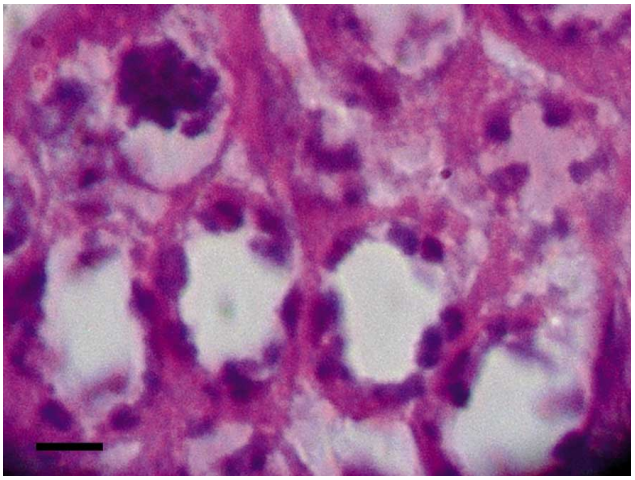

Fig 3b. Malathion-treated group, 33.2 d. Leydig cells karyolysis. (HE, bar = 15µm).

more pronounced at 33.2 days post-treatment (Fig. 2). Leydig cells showed detectable changes by LM as karyolysis, at 33 days post-treatment (Fig. 3). Ultrastructurally, we detected thickening of the basal membrane and vacuolation of Sertoli cells at day 8 (Fig. 4), spermatocytes at the beginning of meiosis were the most affected germ cells (Fig. 5). In Leydig cells, the finding of karyolysis evidenced by light microscopy was confirmed.

Morphometric analysis of seminiferous tubules. The average of the tubules (in all stages of the cycle) increased from 8.3 day post-injection to the day 33.2 animals in control and treated groups, but significantly lower values were shown by the latter ($p < 0.05$) (Fig. 6). An increase of average

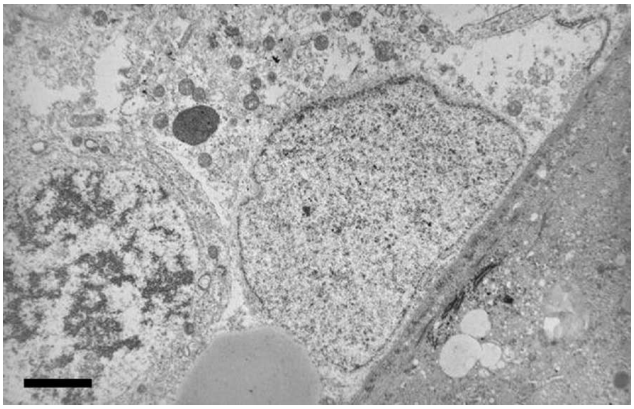

Fig. 4a. Control group. Tubular basal compartment (bar = 50nm).

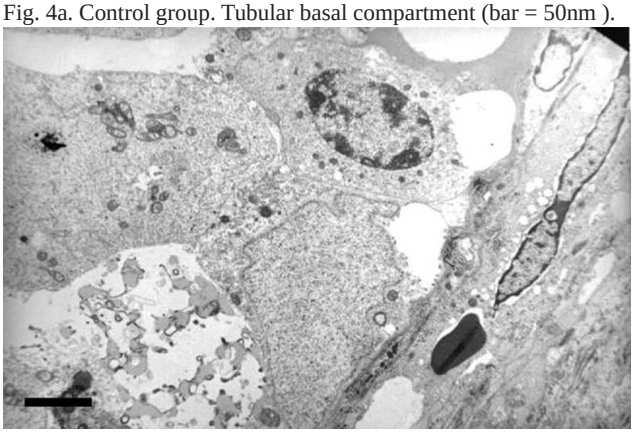

Fig 4b. Malathion--treated group, 8d. Tubular wall thickening, vacuolization of the Sertoli cell and germ cell necrosis. (bar = 50nm).

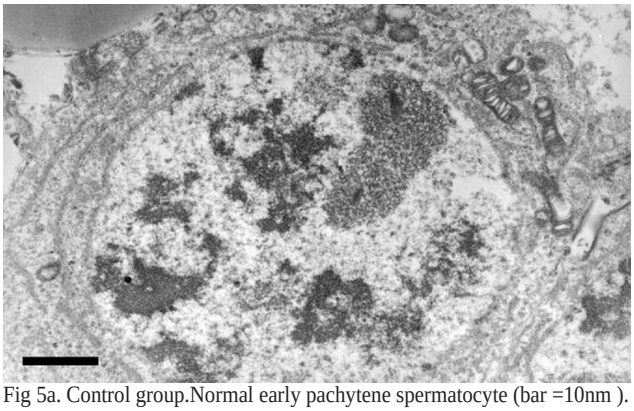

Fig 5a. Control group.Normal early pachytene spermatocyte (bar =10nm).

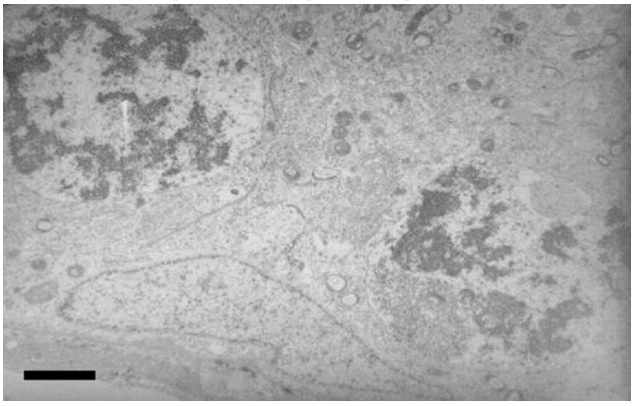

Fig 5b. Malathion treated group, 8d. Karyolisis in spermatocyte (bar =10 nm).

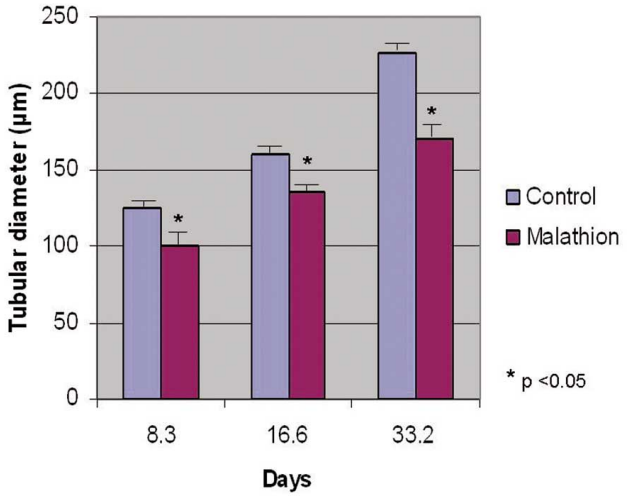

Fig. 6. Tubular diameter (mean ± standard deviation) of control and malathion-treated mice

diameter of tubular stages of the cycle to 16.6 and 33.2 days compared to day 8, with a significant increase of 65% among the group of 16.6 and 8.3 days and 40% among the 33.3 and 16.6 days ($p < 0.05$) was observed. However, there was no difference between the values of the treated and control group for each one of the stages (Fig. 7).

Programmed cell death in seminiferous tubules. In the malathion-treated mice group, there was an increase in the percentage of seminiferous tubules with apoptotic cells, these differences were statistically significant compared to control (Fig. 8, Table I).

Immunohistochemical detection of p53. Was detected in control and malathion treated mice, mainly in pachytene spermatocytes, and immunostaining was more intense in the latter group from 8.3 days (Fig. 9).

Serum testosterone levels and plasmatic acetylcholinesterase activity. Serum testosterone levels decrease significantly at 16.6 days and sustained low at 33.2 days (Table II). Moreover, the plasma acetylcholinesterase activity was reduced in the malathion treated group only 24 hours after administration of the toxicant (Table III).

Blood-testis barrier integrity. Comparing labeling with lanthanum between malathion-treated mice and controls, no differences in indemnity of inter-Sertoli junctions were evidenced.

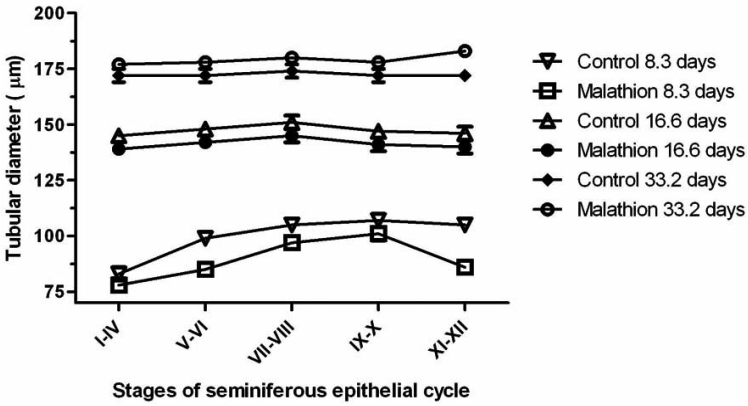

Fig. 7. Tubular diameter (mean± standard deviation) per stage of the cycle of the seminiferous epithelium in control and malathion-treated mice.

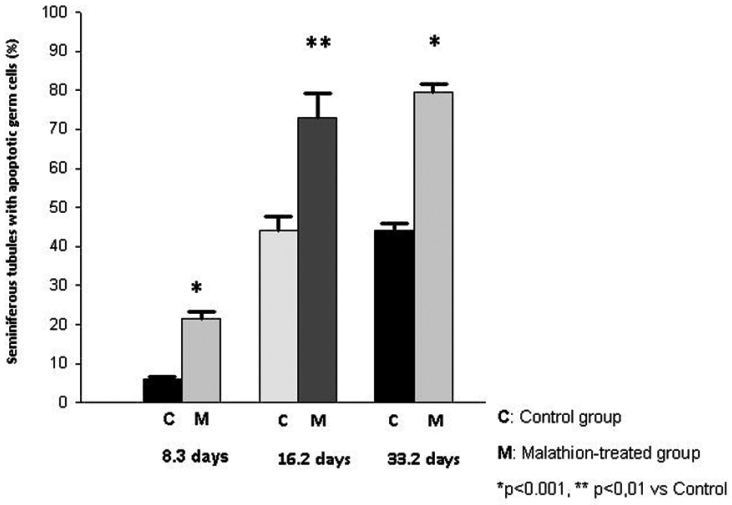

Fig. 8. Percentage of seminiferous tubules with apoptotic germ cells in malathion-treated mice.

Table I. Percentage seminiferous tubules with apoptotic cells in mice after the administration of a sub-lethal dose of malathion.

Days after injection	Groups	
	Control	Malathion (1/12 LD_{50})
8.3	1 ± 1.41	21.5 ± 3.54*
16.6	44 ± 8.48	73 ± 14.14**
33.2	44 ± 4.24	79.5 ± 4.94

Values are expressed as mean ± Standard error (SE) * p<0.00001 versus control ** p< 0.005

Table II. Serum testosterone levels (nmol / L) in mice after administration of a sub-lethal dose of malathion.

Days after injection	Groups	
	Control	Malathion (1/12 LD_{50})
8.3	1.02 ± 0.18	1.08 ± 0.24
16.6	1.68 ± 0.34	1.06 ± 0.07*
33.2	1.75 ± 0.35	1.01 ± 0.06*

Values are expressed as mean ± Standard error (SE) * p<0.05 versus control

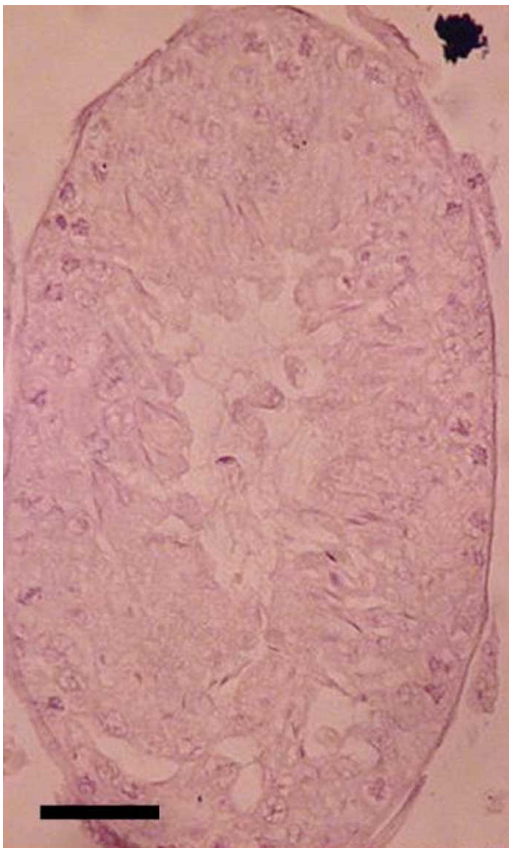

Fig. 9a. Testis, control group. Spermatocytes showing positive staining for p53 (IHC, bar= 100µm).

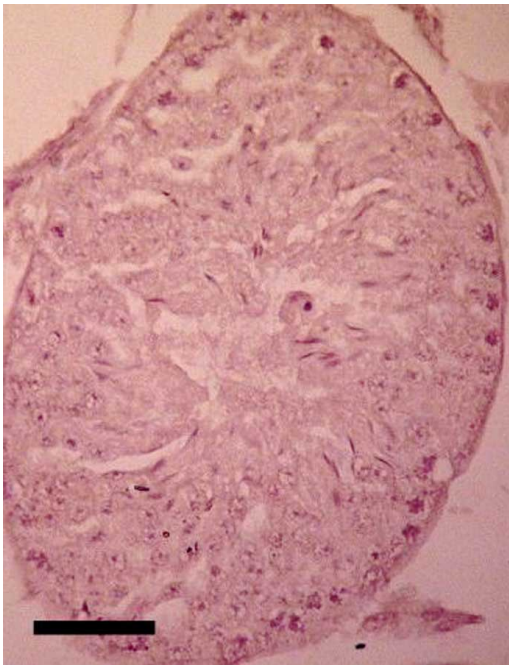

Fig. 9b. Malathion treated group. Pachytene spermatocytes showing intensely positive staining for p53 (IHC, bar= 100µm).

Table III. Serum cholinesterase levels (IU / L) in mice after the administration of a sub-lethal dose of malathion.

Days after injection	Groups	
	Control	Malathion (1/12 LD_{50})
1	4339 ± 930.19	1850.4 ± 72.4 *
8.3	4756.50 ± 436.23	3962.17 ± 698.35
16.6	4458.83 ± 485	4797.5 ± 788.43
33.2	311.2 ± 435.22	4595.4 ± 755.22

Values are expressed as mean ± Standard error (SE) * $p<0.05$ versus control.

DISCUSSION

The results of this study show that administration of an acute sub-lethal dose of MP (1/12 DL50) is able to reduce steroidogenesis, causes apoptosis and induces proliferation of the seminiferous epithelium in mouse.

Morphological alterations in mouse testis were evident by light and electron microscopy, at 8 days after treatment with a single dose of MP, similar to that described by Contreras & Bustos-Obregón (1999). The changes found in spermatocytes corroborates the findings of Bustos-Obregón *et al.*, (1994; 1998), who indicate damage to primary spermatocytes in pachytene stage, involving alterations in the synthesis or DNA repair in late zygotene stage.

The damage to spermatids, in the development of the flagellum which contributes to the lack of maturation of the gametes towards sperm, can also be manifested as reduced sperm count, increased number of abnormal forms in semen or alterations in sperm motility. Our observations indicate that MP can cause damage to spermatids, a fact that has been shown previously and appears to be associated with the properties of OP to interfere with the normal coupling of the proteins that constitute the flagellum during spermatogenesis (Contreras & Bustos-Obregón).

Degenerative ultrastructural findings in the germinal epithelium by action of MP, as found in this study, have been described previously as abnormal patterns of nuclear condensation of elongated spermatids that occur in similar periods at 14 and 26 days post-treatment (Contreras & Bustos-Obregón).

It has been shown that some chemicals, among which OP compounds exert deleterious effects on the division and maturation of male gametes through its alkylating action on proteins and nucleic acids, being considered for that reason as mutagenics (Pandey, 1990; Mathew *et al.*, 1992).

Vacuolization in the cytoplasm of Sertoli cells has been reported as in the present work. This type of injury is an early sign of testicular damage. While the small vacuoles are interpreted as loss of germ cells, the major ones correspond to metabolic alterations of the Sertoli cell (Russell *et al.*, 1990; Nolte *et al.*, 1995, Contreras & Bustos-Obregón, 1999). These changes imply retraction of apical processes of Sertoli cells, affecting specialized intercellular junctions and subsequently releasing the germ cells to the tubular lumen (Russell *et al.*; Nolte *et al.*).

MP reduces sperm count and the number of normal forms, with maximal effect at 18 days post-injection in mice (Bustos-Obregón & González-Hormazábal, 2003). Desquamation of germinal epithelium in the animals treated with MP reflects the reduction of spermatogenesis (Contreras *et al.*, 2006). This has been associated with pseudo-spermiation of morphologically abnormal cells (Contreras & Bustos-Obregón).

These findings indicate that MP affects both somatic and germ cells of the testis and, in addition to the alkylation reactions that occur with proteins and DNA, could also elicit side effects involving extratesticular organs in response to the insecticide (Contreras & Bustos-Obregón; Uzun *et al.*, 2009).

The morphometric analysis is consistent with that described by Wing & Christensen (1982) in the rat. The tubular diameter is dependent on the stage. During the first interval (8.2 and 16.6 days post-injection) minimal differences between control and treated groups and associations that are not complete were seen. The difference is more evident at 33.2 days. It can be deduced that MP alters the normal growth and development of cell cycle stages associations in the early periods of spermatogenesis.

Reduced testosterone levels were found by 16.2 days and were maintained at 33.2 days. The histopathological findings revealed nuclear alterations in Leydig cells of animals treated with the pesticide. This result coincides with those of Bustos-Obregón & González-Hormazábal indicating a toxic effect of MP on the Leydig cells which, like other OP (Contreras *et al.*), inhibits non-specific esterase activity of these cells, reducing the production of male steroids (Chapin *et al.*, 1990).

MP also affects, per se, testosterone levels in rats, without altering LH values (Krause). Furthermore, testosterone, through modulation of the peritubular cells P-mod-S, affects the function of Sertoli cells (Skinner & Fritz, 1985) that may

contribute additionally with the desquamation and depletion of germinal epithelium in the seminiferous tubules.

Atef *et al.*, (1995) demonstrated the deleterious effect of phoxim, an anti-cholynesterase pesticide on the testosterone synthesis. Krause *et al.,* (1975) and Krause (1977) indicate that MP causes damage to the Leydig cells and reduces testosterone levels. This interferes in the process of spermatogenesis, by preventing the maturation in the later post-meiotic stages, which are androgen-dependent (Russell *et al.*). Also, Krause *et al.,* have described reduction in the number of immature germ cells, as a consequence of decreased steroidogenic activity and damage of the Sertoli cells.

The effect on the steroidogenic function has been explained by alkylating cytotoxic action exerted by OP on the steroidogenic cells (Krause *et al*.). Some OP inhibit transcription of the protein involved in a limiting enzymatic step, as the transfer of cholesterol from the outer to the innner mitochondrial membrane (Walsh *et al*., 2000).

The determination of plasma cholinesterase has been used to detect exposure to OP. As described by Bustos-Obregón & González-Hormazábal, with the exception of day 1 post-injection, no differences were found between treated and control animals, indicating that the pesticide does not induce a state of general intoxication but provokes other local changes in the testis, as evidenced in the desquamation of germinal epithelium and therefore in the reduction of spermatogenesis.

Administration of a sub-lethal dose of MP in albino mice induced a progressive increase in the number of apoptotic germ cell, from the first spermatogenic cycle, throughout spermatogenesis. However, recovery was observed at 16 days, which may be related to the division of spermatogonia that proliferate responding at 2 weeks post-treatment, a mechanism that has been already described (Thumann & Bustos-Obregón, 1978). Other pesticides such as parathion and paraoxon, have been also identified as inducers of apoptosis in the germinal epithelium (Rodriguez & Bustos-Obregón).

It seems likely that MP has caused damage to the testis by two cell death mechanisms: one mediated by apoptosis, in the case of germ cells and other cells and by necrosis of Leydig cells. Henriksen *et al.* (1995), studying the effects of ethanol dimethanol sulfonate (EDS), a toxicant for Leydig cells in the epithelium of adult rats concluded that a decrease of testosterone induces cell death by apoptosis. These and other observations suggest the hypothesis that DNA is a target for toxic cell death.

Apoptosis plays an important role in the development of testicular germ cells and appears to be a mechanism responsible for male infertility caused by high temperature, toxicants, radiation, chemotherapy, hormone depletion and testicular torsion. In cryptorchidism, for instance, it has been shown in mouse that a p53-dependent pathway is responsible for the initial portion of the germ cell loss by apoptosis 7 to 9 days after exposure to high abdominal temperatures, and the subsequent apoptosis involves an unknown route, independent of p53 (Yin *et al*., 1998).

The tumor suppressor protein p53, also called guardian of the cell cycle has important functions in growth and cell differentiation. After DNA damage caused by radiation, p53 is usually regulated additively in mammalian cells resulting in apoptosis initiation, repair pathways and cell blockage. Particularly during spermatogenesis, the mRNA and protein p53 are present in primary spermatocytes. After exposure to some insults such as radiation, p53 expression is enhanced in these cells. It is believed that p53 is important in regulating cell production during normal spermatogenesis either by regulation of cell proliferation or, more likely, by regulation of apoptosis in spermatogonia. In addition, it is involved in removing severely damaged spermatogonia (Beumer *et al*., 1998)

During normal spermatogenesis, mouse spermatogonia do not express p53, when analyzed by immunohistochemistry. However, after exposure to radiation, p53 is demonstrated in spermatogonia, suggesting that it plays an important role in normal spermatogenesis and regulation of apoptosis following DNA damage (Beumer *et al*.; Ohta *et al*., 2003). In the present study, p53 is mostly expressed in pachytene spermatocytes as a proliferative compensatory response to an increased apoptosis, caused by MP.

Testicular toxicity appears early after the administration of MP (8.3 days), a fact also observed with other toxicant agents (Matsui *et al.*, 1995; Spanó *et al.*, 1996). The effect of MP appears to be reversible and of short duration, because at 33.2 days spermatogonia and spermatocytes are recovered (Bustos-Obregón & Sobarzo). In conclusion, the findings of this study and those described in the literature indicate that MP alters the testicular function affecting the DNA. Under the conditions of this study MP interferes with both spermatogenesis and steroidogenesis.

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RESUMEN: La restricción de los mecanismos de proliferación celular en epitelio seminífero murino, en términos de inducción de muerte celular programada hasta hace poco no había sido completamente analizada. El objetivo de este trabajo fue evaluar el efecto de malathion (MP) sobre la morfología y la función testicular del ratón. Ratones macho albinos de la cepa NMRI-IVIC, con pesos entre 30-40 g fueron utilizados, se dividieron en grupos control y experimental. Los grupos experimentales fueron inyectados por vía intraperitoneal con una dosis única de MP: 241mg/kg de peso (1/12 DL 50) resuspendido en 0,9% de solución salina. Los animales fueron sacrificados en el día 8,3, 16,6 y 33,2 después de la inyección (primer, segundo y tercer ciclos de la espermatogénesis). Se obtuvieron muestras de testículo para estudio en microscopía de luz (ML), microscopía electrónica de transmisión, para la detección de apoptosis y el antígeno p53 (proliferación celular), por métodos inmunohistoquímicos. Se recogió sangre para cuantificar la testosterona y la actividad plasmática de colinesterasa. Desde el día 8,3 día se observó vacuolización de células de Sertoli, cariolisis de espermatocitos en paquiteno y células de Leydig, y una disminución en el promedio del diámetro de los túbulos seminíferos. No se detectó daño en las uniones entre células de Sertoli. El porcentaje de túbulos seminíferos que mostraban células germinales en apoptosis se incrementó a los 8,3 días, la actividad de la acetilcolinesterasa plasmática se redujo en el grupo tratado sólo 24 horas después de la administración de MP. Los niveles séricos de testosterona disminuyeron en los animales tratados a los 16,6 y 33,2 días. P53 se expresó sobre todo en los espermatocitos en paquiteno desde los 8,3 días. Los resultados de este estudio indican que MP altera la función testicular, afecta al ADN e interfiere con la espermatogénesis, así como con la esteroidogénesis.

PALABRAS CLAVE: Malathion; Testículo; Ratón; Apoptosis; Morfología; p53; Proliferación.

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