Testosterone production and spermatogenic damage induced by organophosphorate pesticides

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ABSTRACT: Parathion® is an organophosphorate pesticide amply used in agriculture. Many alterations induced by organophosphorate pesticides have been described, such as: cytogenetic alterations in germinal cells, oligozoospermia and teratozoospermia in the mouse. The effect of Parathion®, both pure (PP) and commercial (PC), on mouse interstitial cell testosterone production was evaluated *in vivo* and *in vitro*. Male mice were intraperitoneally injected with a single dose of 1/3 LD50 of Parathion®, both PP and PC. The animals were sacrificed at 1, 8 and 40 days post injection to evaluate the impact of disrupting testosterone production on spermatogonia, spermatocytes and elongated spermatids. The plasma testosterone was assayed by standard radioimmunoanalysis. The same method was used to assay testosterone in the culture medium of interstitial cells obtained from the control and Parathion® treated animals at the same time intervals. Sperm count, sperm teratozoospermia and tubular blockage were analyzed for an appraisal of spermatogenesis. Increase in the teratozoospermia and tubular blockage was detected in the PP and PC group at 8 and 40 days post injection. Plasma testosterone levels drop significantly at 8 days and recovered slowly at 40 days only in PP animals as detected *in vivo,* implying interference of testicular steroidogenesis due to the toxicant. Recuperation of normality occurs at long time intervals. In conclusion, Parathion® disturbs the synthesis of testosterone in mice affecting qualitatively the spermatogenesis

Introduction

The key role of testicular testosterone for normal spermatogenesis has been clearly established (Steinberger, 1971). The testicular steroidogenesis can be interfered by many exogenous factors (chemicals, drugs, etc) acting in many different ways due to the complex physiological mechanisms that regulate Leydig cell function (Cooke, 1998). As a consequence of impaired Leydig cell activity, male infertility may result (Payne *et al.,* 1980).

Spermatogenesis is a process under hypophyseal hormonal control that involves gonadotropin synthesis of LH and FSH. These hormones act on Leydig and Sertoli cells respectively (Steimberger, 1971). Also interactions between Leydig cells-Sertoli cells are necessary for normal intratesticular testosterone production (Skinner, 1991). The Leydig cells produce testosterone needed in the seminiferous tubules to induce the differentiation of spermatogonia to spermatozoa. Alterations in the testosterone synthesis could be due to many different pathological or experimental situations (Dufau *et al*., 1979). Intratesticular testosterone levels that promote normal spermatogenesis are 100-fold

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higher than those found in blood plasma and they are accomplished through normal Leydig and Sertoli cell activity (Dufau *et al*., 1979; Sáez *et al.,* 1987; Russell *et al*., 1998).

The widely used organophosphorate pesticides, are esters of phosphoric (or thiophosphoric) acid. Although they are rapidly metabolized, they are highly toxic for insects and mammals. The main chemical pesticide of this group is Parathion®. In mammals, it is metabolized by oxidation, hydrolysis and demethylation generating paraoxon, which is highly toxic (Levi, 1987). The toxic action of Parathion® is due to its interference with the active site of the enzyme acetylcholinesterase, which is blocked by phosphorylation (Guillermino *et al*., 1998).

It has been described that organophosphorate pesticides utilized in the control of plagues such as Parathion®, Malathion®, Diazinon® and others, have adverse effect on reproductive health (Rojas *et al.,* 1998). Alteration of germinal cell DNA, seminiferous epithelium, teratozoospermia, azoospermia and morphofunctional properties of sperm have been previously reported (Sobarzo and Bustos-Obregon, 2000; Contreras and Bustos-Obregón, 1999; Rodriguez and Bustos-Obregón, 2000; Contreras *et al.,* 1999; Bustos-Obregón and Gonzalez-Hormazábal, 2003; Del Valle *et al.,* 2004; Espinoza-Navarro and Bustos-Obregón, 2005). Some *in vivo* effects of organophosphorate pesticides on reproductive parameters have been analyzed. At a single dose, the subcutaneous application of organophosphorate pesticides induced teratozoospermia and ultraestructural damage in the cytoplasm of Sertoli cells in mice. Effects of organophosphorate pesticides on steroidogenesis were unclear (Contreras and Bustos-Obregón, 1999).

To assess the effects of Parathion® on male mice reproductive physiology, testosterone production and spermatogenesis were evaluated *in vivo* and *in vitro*. Intervals of sacrifice of the animals were selected to evaluate the impact of disrupting testosterone production on spermatogonia, spermatocytes and elongated spermatids, as judged by epididymal sperm count and morphology.

Materials and Methods

Animals

Male CF1 strain mice (70-80 day old, average weight 32 g) were fed with commercial pellets, given water *ad libitum*, and kept with a photoperiod of 12:12 hour light darkness. The animals were intraperitoneally injected with a single dose (1/3 LD 50) of either commercial Parathion® (PC, ANASAC, Santiago, Chile. 80% w/v) or pure Parathion® (PP,O,O-diethyl O-4-nitrophenyl phosphorothioate, Sigma, USA. 99.6% w/v). Doses utilized were 9 mg/Kg body weight of PC and 300 mg/Kg of PP. Controls were injected with physiological solution (0.85% NaCl). Each group of mice comprised 5 animals per treatment and time interval (60 mice in total) that were sacrificed 1, 8 and 40 days after injection. Timing was selected to evaluate the acute Parathion® effect (day 1), and damage elicited on the following germ cell populations: elongated spermatids (day 1), primary spermatocytes (day 8) and spermatogonia (day 40).

Plasma acetylcholinesterase activity

Heparinized blood samples were obtained through cardiac puncture and the plasma kept at -20ºC until analysis. The acetylcholinesterase activity was determined according to Rappaport *et al*. (1959) using a Sigma Diagnostic colorimetric kit (St. Louis, USA). These assays detect a pH change (read at 420 nm) due to acetic acid produced by hydrolysis of acetylcholine. One Rappaport unit is defined as μmol of acetic acid produced by acetylcholine hydrolyzed in 30 min at 25ºC and pH 7.8.

Sperm count

Epididymal spermatozoa were obtained as previously described by Sobarzo and Bustos-Obregón (2000). The epididymis were weighed, macerated in Petri capsules with a volume of 3.0 ml phosphate buffer saline, filtered

TABLE 1.

Sperm Count expressed as 10^6 of spz/mg epididimys in mice injected with pure Parathion® (PP) and commercial Parathion® (PC).

	C	PP	PC
1 day	167 ± 12	122 ± 14	162 ± 11
8 days	144 ± 9	142 ± 6	77 ± 13*
40 days	149 ± 10	96 ± 13*	135 ± 10

Mean ± SD. *p<0.005 vs. control (Fisher test).

and resuspended. Sperm counts were done using a Neubauer hemocytometric chamber. The results were expresses as millions of sperm per milligram of epididymis.

Sperm morphology

Washed sperm were utilized for morphological analysis. Hematoxylin-eosin stained smears were used for light microscopy evaluation (1000x). Cell counts were expressed as 10^6 spermatozoa per mg of weight fresh epididymal. Teratozoospermia followed the criteria described for mouse spermatozoa (Vigil and Bustos-Obregón, 1985). Spermatozoa that presented a combination of head and tail anomalies were classified according to the most evident abnormality.

Sperm chromatin stability

The thermal pattern of DNA denaturation was tested by incubating sperm samples at 90º for 0, 2 and 10 minutes. The sperm smear was then stained with acridine orange according to Tejada *et al.* (1984). Normal chromatin stains orthochromatic, while denatured nuclei stain metachromatic as seen under a fluorescence microscope (Zeiss MO1) at 524 nm.

Testicular histopathology

The testicular histopathology was evaluated to previously criteria (Russell *et al*., 1990; Sobarzo and Bustos-Obregón, 2000). The right testes was fixed in alcoholic Bouin for 8 h, embedded in paraffin, cut and stained with PAS- Hematoxylin. Fifty cross sections of seminiferous tubules per animal were observed at 1000x and digitalized to measure epithelial height and tubular diameter using a computer program (Scion Image beta 3b, NIH, USA). The percentage of tubules with luminal blockade was determined using a microscope.

In vitro production of testosterone

Control and treated group underwent cardiac puncture to obtain blood plasma for testosterone assay. They were sacrificed and thereafter their decapsulated testes were incubated in minimal essential medium (MEM) with 5% BSA added. Crude preparations of interstitial cells were obtained by enzymatic digestion with 1% collagenase, in a bath at 37ºC for 10 min with continuous shaking. Cells were washed afterwards with MEM and incubated for 2 h at 37ºC in an atmosphere of 5% CO2 and 95% air. After 2 h, the culture was put in Kahn tubes and heated for 5 min in a bath at 100ºC. After heating, the tubes were centrifuged at 800 g for 5 min and the supernatant was frozen at -20ºC until testosterone assay by RIA was done using the same kit described below. The percentage of Leydig cells that were present in these assays was estimated by the number of 3ß -hydroxysteroid dehydrogenase stained cells, a key enzyme in the steroidogenic pathway and only present in Leydig cells. The cells obtained were 63% positive stained and their viability higher than 89% as previously described by Contreras and Ronco (1994). Data were expressed as ng/ml for blood testosterone and as ng/100.000 interstitial cells for *in vitro* assays.

Blood plasma testosterone assay

At least 1 ml of blood was obtained from each mouse by cardiac puncture. After clotting, blood serum was separated by centrifugation at 1000 g fot 10 min and kept frozen at -20ºC until testosterone assay by RIA

TABLE 2.

Teratozoospermia (%) in mice injected with pure Parathion® (PP) and commercial Parathion® (CP).

	Normal		Anomalies			
			Head		Tail	
	8 days	40 days	8 days	40 days	8 days	40 days
C	87.1 ± 4	87.1 ± 4	3.3 ± 1	3.3 ± 1	8.9 ± 2	8.9 ± 2
PP	71.6 ± 5**	62.3 ± 3**	6.9 ± 2*	6.6 ± 3*	21.6 ± 3*	32.6 ± 4*
PC	74.3 ± 4**	54.3 ± 2**	5.9 ± 2*	2.8 ± 1	20 ± 2*	42.7 ± 4**

Mean ± SD. *p<0.005 and **p<0.001 vs control (Fisher test).

of solid phase using a commercial kit (Coat a Count, DPC; USA) and I^{125} as tracer.

Statistical analysis

Statistical analysis was performed by ANOVA, Fisher test for comparisons of control and treated group and Student-Neuman-Keuls test for multiple analyses. Significance was set at $p< 0.001$ and $p< 0.005$.

Results

Plasma Acetylcholinesterase activity

Plasma acetylcholinesterase activity was affected at 1 day post injection with Pure Parathion® (PP), decreasing from 108 Rappaport unit/ml to 56 Rappaport unit/ml. The group injected with Commercial Parathion® (PC) had 116 Rappaport unit/ml, similar to control values at the same period. At 8 days post injection the values were 95 and 103 Rappaport unit/ml for PP and PC, respectively. The values at 40 days post injection were 92 and 98 Rappaport unit/ml for PP and PC respectively; therefore, they are not significantly different from those from the control group. The intoxication with Parathion® was only evident at 1 day post injection (acute effect) but it was not a critical condition for the health of experimental mice in any case.

Sperm count

Cell counts showed no differences at 1 day post injection. There was a decrease in the spermatic count at 8 days only in the PC group and 40 days in the PP group. The damage induced at 8 days for PC might be related to different solvent used in the commercial formulation as xylene and other organic solvent (Table 1).

Teratozoospermia analysis

Analysis of the teratozoospermic spermatozoa revealed that head and tail anomalies were significatively increased in PP and PC groups as compared with control animals (Table 2). PP (6.9 ± 2) and PC (5.9 ± 2) showed similar effect at day 8 when abnormal head were evaluated. Tail anomalies increased in both PP (21.6 ± 3) and PC (20 ± 2) groups. The teratozoopermia was higher at 40 days post injection to value 32.6 ± 4 and 42.7 ± 4 in the PP and PC groups, respectively, suggesting that the populations more sensitive to the pesticide were early spermatogonia (Table 2). At day 1 post injection no teratozoospermia was recorded.

Sperm chromatin stability

The values of orthochromatic sperm decreased after 2 and 10 min of thermal denaturation. At 8 and 40 days post injection the differences were largest at all times studied in both treated groups (Table 3). At 1 day no significative differences were observed in all groups studied.

TABLE 3.

Orthochromatic sperm (%) in mice injected with pure Parathion® (PP) and commercial Parathion® (PC).

		Control	P P	P C
1 day	0 min	100	100	100
	2 min	99	91.9	100
	10 min	58.6	48.6	68
8 days	0 min	100	52.9*	1.8**
	2 min	98.3	33.4**	0.05**
	10 min	58.6	5.4**	1.8**
40 days	0 min	98.9	81.3*	86.6*
	2 min	90.1	13.5**	63.8*
	10 min	55.2	3.2**	17.4**

Mean (%). *p<0.05 and **p<0.01 vs control (Fisher test).

TABLE 4.

***In vitro* production of testosterone (ng/100.000 cell culture) in interstitial cell culture of CF-1 mice 1, 8 and 40 days after a single injection of pure Parathion® (PP), commercial Parathion® (PC) or physiological solution (C, control).**

	1 day	8 days	40 days
Control	10.02 ± 2	14.09 ± 0.3	14.809 ± 1.2
PP	12.14 ± 3.6	11.03 ± 0.3	14.502 ± 1.9
PC	11.754 ± 3.7	10.73 ± 0.23	12.574 ± 1.3

Mean ± SD non difference significant for $p < 0.01$ vs control (Fisher test).

Testicular histopathology

Analysis of testicular histopatology showed a significative increase in the tubule blockage at 8 and 40 days post injection in the PP and PC group (Fig. 1A and B, respectively). At 40 days the value increased up to 26.1% and 24.8% in PP and PC groups, respectively, compared to control value (4%). At 1 day post injection no significative differences were observed (data not show).

Basal in vitro testosterone production

Basal *in vitro* testosterone production by interstitial cells of control and Parathion® treated mice did not differ for animals sacrificed 1 day after injection though there was a tendency (non significant) of testosterone production to decrease by 8 days, mostly in PC samples. Similarly the *in vivo* tendency, at 40 days, is the same as controls, concerning testosterone production for the PP samples while testosterone in PC samples remains low (Table 4).

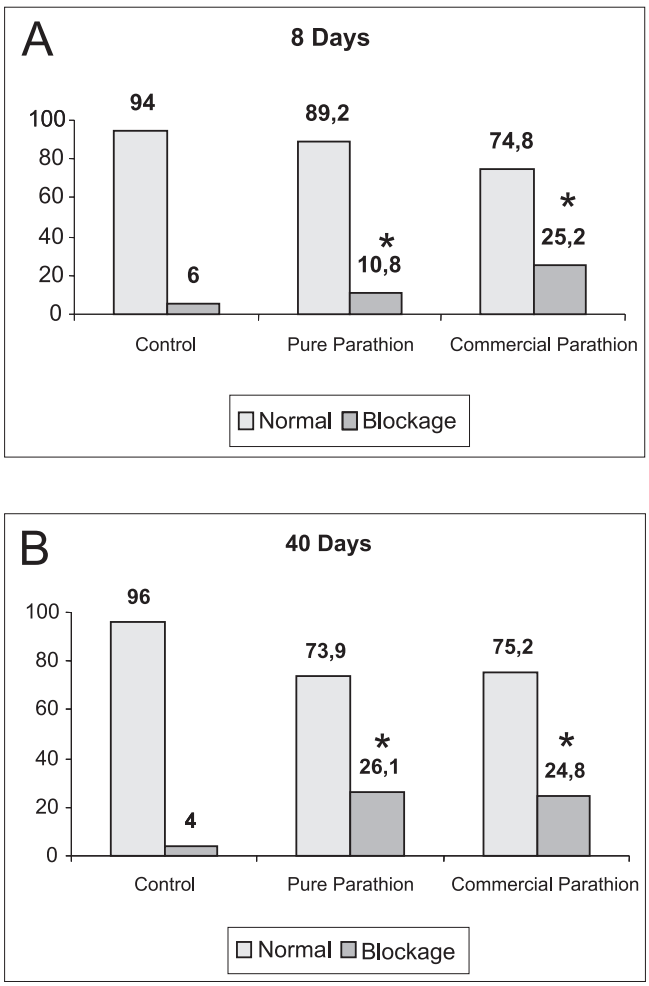

FIGURE 1. Tubular blockage in testes of CF-1 mice 8 (A) and 40 (B) days after a single injection of pure (PP) or commercial (PC) Parathion® or physiological solution (C, control). Data are expressed as the mean ± SEM, n=5 (*p < 0.01).

Serum testosterone levels

One and eight days after injection (Table 5), there was a significant decrease in blood testosterone levels in mice treated with either PP or PC. The effect of both organophosphoric toxics was very similar.

At longer time intervals after injection, blood testosterone levels differ for both treatments. There is a marked increase of testosterone concentration in the PP treated mice while it remains low (as after 1 or 8 day after injection) in the PC treated animals 40 days after injection (Table 5). The opposite is found by 50 days with testosterone levels of PP treated mice, much lower than those found in PC treated animals which are 4-fold higher than controls (data not shown).

TABLE 5.

Testosterone level (ng/ml) in serum of CF-1 mice 1, 8 and 40 days after a single injection of pure Parathion® (PP), commercial Parathion® (PC) or physiological solution (C, control).

	1 days	8 days	40 days
Control	3.3 ± 0.2	3.27 ± 0.23	3.19 ± 0.1
PP	0.45 ± 0.31	0.3 ± 0.09*	8.23 ± 2.9
PC	0.33 ± 0.21	0.21 ± 0.1*	0.23 ± 0.9*

Mean ± SD. *p < 0.01 vs control (Fisher test).

Discussion

Testicular Leydig cell is the main site of testosterone synthesis. This steroidal hormone plays a key role in maintenance of spermatogenesis, male sex characteristics and fertility. A wide range of agents is known to alter Leydig cells in rat, including numerous chemicals, some of them agropesticides of the organochlorine type (Cooke, 1998). Some of these environmental toxics may cause Leydig cell tumors in rat and mice, though in the latter the incidence with ageing is only 1-2% after 8 months of age (and none in young adults).

Plasma acetylcholinesterase level is a useful clinical method to evaluate exposure to organophosphorate pesticides (Nigg and Knaak, 2000). A 50% decrease 1 day after Parathion® injection implies that there was an acute effect in mice under our experimental conditions (Bustos-Obregón and Gonzalez-Hormazábal, 2003).

The sites of action of testicular toxicants acting on Leydig cells might be many, since the normal pathway of testosterone production involves hypothalamic secretion of GnRH, stimulation of the adenohypophysis by GnRH to produce LH and the interaction of LH with Leydig cells to stimulate testosterone production, which in addition has a paracrine regulation by Sertoli cell-derived estradiol and GnRH (Bustos-Obregón and Gonzalez-Hormazabal, 2003). Moreover, testosterone secretion is also influenced by plasma prolactin levels and cytokines produced by macrophages (Morris, 1996).

Our observations on blood testosterone levels following injection of an organophosphoric pesticide indicate that Leydig cell steroidogenesis is acutely and deeply damaged by Parathion®, blood testosterone drops at 8 days after administration of the toxicant. Recuperation of testosterone production, with an overshooting over control levels is obtained only 40 days for PP treated animals. These are very long intervals in terms of mouse spermatogenesis, since its full duration is 35 days (Oakberg, 1956). The disruption of steroidogenesis under our experimental condition is evident and this result is in agreement with data previously reported (Bustos-Obregón and Gonzalez-Hormazabal, 2003).

Low blood testosterone levels for long intervals may result in spermatogenic deficiencies. In fact, lowered sperm counts in the epididymis, increased teratozoospermia and chromatin defects of sperm nuclei have been reported in mice after Parathion® treatment (Guillermino *et al.,* 1998). The qualitatively decrease in the spermatogenesis might to explained by tubular blockage observed in the testes morphology associated with low level of testosterone.

Basal (non stimulated) *in vitro* testosterone release to the medium by interstitial cells of the mice did not show acute impairment of androgen production in contrast to the *in vivo* observations. There was only a tendency to a decreased production with both PP and PC. These results suggest that the dramatic effects observed *in vivo* might be due to interference of the toxicant with the hypophysis modifying the release of LH, or altering the response of Leydig cells to LH. The cascade of events may be as complex as those reported for the effect of dopamine agonists on Leydig cell function. To answer satisfactorily this hypothesis more experiments are necessary (Cooke, 1996).

After 50 days, only PP samples recuperate *in vitro* the ability to secrete testosterone with a marked overshooting compared to controls which may imply the lack of inhibitory signals (data not shown). Some of them may be originated in the Sertoli cells that are known to undergo prolonged damage by organophosphoric compounds (Rojas *et al*., 1998; Bustos-Obregón and Gonzalez-Hormazabal, 2003). It is very important to consider that interstitial cells culture when stimulated with hCG, do not show increased response in testosterone synthesis, as it would be expected and effect previously described (Contreras and Ronco, 1994). The eventual selective damage on Leydig cell requires confirmation. Further insight on the way agropesticides act on Leydig cell function requires *in vitro* analysis of steroidogenesis and the effect that these toxicants have on enzymatic pathway of testosterone synthesis as well as on general detoxifying mechanisms of the cells, such as the cytochrome P-450 complex (Butler and Murray, 1993).

In conclusion, a single dose of Parathion®, pure or commercial formulation, elicits an alteration on steroidogenesis and spermatogenesis at long time. The testosterone synthesis affects the differentiation of germinal cells at the primary spermatocytes (days 8) and spermatogonial (40days) stages but not at elongated spermatids (day 1).

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