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Paraoxon Inhibits Fertilization of Mouse Gametes *In Vitro*

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Potential exposure of mammals to organophosphate pesticides and their metabolites is recognized (Frank *et al.* 1991). Organophosphates have been implicated frequently in suboptimal reproductive performance in males and females of various species. Studies have shown that altered androgen metabolism (Krause, 1977), pathological changes in testes and adrenals (Chapin *et al.* 1988; Oishi *et al.* 1982; Dunnick *et al.* 1984), and chromosome aberrations (Kiraly *et al.* 1979) may be responsible for decreased reproductive performance in animals exposed to organophosphates. Also, organophosphates may have direct effects on sperm fertilizing ability without producing organic changes or altering spermatogenesis (Beck 1953; Harbison *et al.* 1976). This effect on sperm may occur in the epididymides, seminal plasma, or female reproductive tract.

There are limited studies of mechanisms by which organophosphates affect fertilization in mammals. The objective of this work was to determine whether or not organophosphates are toxic to the sperm and/or oocyte.

MATERIALS AND METHODS

Glutaraldehyde, human chorionic gonadotropin (hCG), pregnant mare serum gonadotropin (PMSG), lacmoid, malic acid, penicillin potassium, streptomycin sulfate, sodium pyruvate, and TRIS were from Sigma Chemical Co. (St. Louis MO), Brinster's Medium (BMOC-3) (Brinster 1971) was from Gibco (Chagrin Falls OH), and paraoxon was from Aldrich Chemical Co. (Milwaukee WI). Paraoxon was repurified according to Johnson (1977). Two inbred strains of mice (C57BL-6J females and DBA-2J males) were purchased from Jackson Laboratory (Bar Harbor ME). Gametes were obtained from the first generation cross (B6D2-F1). Mice were maintained at 24°C and 14 h -10 h light dark cycle. Mouse Breeder Blox (Wayne Pet Food Division, Chicago IL) and water were provided *ad libitum*.

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Sperm were obtained from 4-7 month old B6D2-F1 males by mincing cauda epididymides in 0.5 ml BMOC-3. The sperm suspension was diluted 1:13 and incubated at 37°C from 0 to 3 h prior to insemination. Sperm motility was determined using a hemocytometer. Oocytes were prepared by superovulating 25 day-old B6D2-F1 females using intraperitoneal injections of 8 IU PMSG and 8 IU hCG 48 h apart. Twelve to 15 h after the last injection oocytes in cumulus mass were recovered by flushing oviducts with 0.5 ml of BMOC-3. Oocytes were then diluted with 1.8 ml of BMOC-3. Usually insemination was with 0.2 ml sperm giving a final sperm concentration of 2-4 x 10^6 sperm/ml of media. Gametes were incubated under 5% CO_2 in air at 37°C and 100% humidity.

Oocytes were harvested 24-27 h after insemination washed with 2 ml of BMOC-3 by micropipetting and fixed overnight in 0.5 ml and 2 ml, respectively, of 2.5% glutaraldehyde and 10% neutral formalin in phosphate buffer, pH 7.0. Oocytes were dehydrated on a glass slide by adding in series 50, 75, and 95% ethanol solutions followed by staining with 0.5% lacmoid acid in 45% acetic acid. Oocytes at the 2-cell stage or containing a second polar body and two pronuclei were considered fertilized.

The effects of organophosphates on sperm motility were studied by collecting sperm in media containing the organophosphate. Motility was measured after 1 h incubation.

To determine whether paraoxon directly affects oocytes prior to insemination, oocytes were collected in media containing 50 μM paraoxon and incubated 1 h. One aliquot of oocytes was washed free of paraoxon. Oocytes were then inseminated with sperm preincubated 3 h in paraoxon-free media.

To study direct effects of paraoxon on sperm, sperm were preincubated with 50 μM paraoxon for 1, 2, or 3 h prior to insemination. In all cases, sperm were incubated a total of 3 h. For one treatment sperm were used immediately after collection and in one treatment paraoxon was diluted to 5 μM before insemination.

Results from each test were recorded in contingency tables as fertilized or unfertilized oocytes. Chi-square statistics were applied to test the hypothesis that fertilization was independent of treatment effects. For experiments involving more than two treatments, rejection of overall hypothesis of independence was followed by comparison of two treatments at a time using Bonferroni critical values. The probabilities of type I error from two or more independent tests were combined using Fisher's Chi-square addition theorem (Gill 1978). The ratio of fertilized to

unfertilized oocytes is expressed as percent fertilized.

RESULTS AND DISCUSSION

Initial sperm motility (about 60%) did not change after one h incubation and was not affected by malathion, parathion, or paraoxon (Table 1).

Table 1. Effects of incubating mouse sperm in 50 μM malathion, parathion, or paraoxon for 1 h on motility.

	Treatments[a]			
	Control	Malathion	Parathion	Paraoxon
No. of experiment	25	3	7	9
Motility (%)	66(11)	62(19)	60(16)	64(12)

[a] Numbers in parenthesis are standard errors.

At 50 μM, neither malathion, parathion, nor DFP affected fertility of oocytes or sperm (Table 2). However, 50 μM paraoxon reduced oocytes fertilized to 37% of controls (Table 2). Similarly 5 μM paraoxon reduced the value to 68% of controls.

Results of studies to determine if effects of paraoxon on fertilization are on the oocytes only are in Table 3. When oocytes were incubated 1 h in 50 μM paraoxon and then washed free of the chemical prior to insemination, fertilization was enhanced. When oocytes were incubated in 50 μM paraoxon both before and after insemination, fertilization was not affected. For these studies sperm were incubated in normal media without paraoxon for 3 h prior to insemination. This experiment shows that paraoxon does not affect fertility of oocytes inseminated by fully capacitated sperm. Consequently, toxic effects are on the sperm.

To determine how long sperm must be exposed to paraoxon before fertilization is affected, paraoxon was added to the incubation media such that sperm were exposed to paraoxon 0, 1, 2, or 3 h prior to insemination. Paraoxon (50 μm) added at insemination, reduced fertilization by 17%. However, adding paraoxon 1, 2, or 3 h before insemination reduced fertilization by 40 to 50% (Table 4).

Paraoxon but not malathion, DFP, or parathion reduced fertilization. It appears to inhibit fertilization by affecting sperm and not oocytes.

Parathion is known to inhibit fertilization in sea urchin gametes (Ferrori et al. 1989). They appear to be more susceptible to parathion than mouse

Table 2. Effects of malathion, parathion, diisopropyl fluorophosphate (DFP), and paraoxon on fertilization of mouse gametes *in vitro*. Sperm were incubated in media containing the test chemicals for 1 h prior to insemination.

Experiment no.	Replicates	Oocytes inseminated	Oocytes fertilized (%)[a]
I: Control	3	76	68(11)[b]
Malathion (50 μM)	3	76	57(5)[b]
Parathion (50 μM)	3	131	53(2)[b]
II: Control	5	89	72(11)[c]
DFP (50 μM)	5	129	56(9)[c]
III: Control	15	459	60(7)[d]
Paraoxon (50 μM)	15	531	22(5)[e]
IV: Control	3	150	73(1)[f]
Paraoxon (5 μM)	3	124	50(17)[g]

[a] Numbers in parenthesis are standard errors.
[bcdefg] Numbers within each experiment with different superscripts differ ($P < .001$).

Table 3. Effects of treating mouse oocytes with paraoxon (50 μM) for 1 h before or both before and after insemination on fertilization *in vitro*. Sperm were incubated 3 h in paraoxon-free-media prior to insemination.

Treatment	Oocytes inseminated	Oocytes fertilized (%)[a]
Control	82	62(8)[b]
Paraoxon (1 h pretreatment)	108	80(7)[c]
Paraoxon (continuous treatment)	82	62(8)[b]

[a] Numbers in parenthesis are standard errors.
[bc] Means with different superscripts are different ($P < 0.1$).

gametes. In both species inhibitory effects depend on pretreatment of sperm. Bishop *et al.* (1976) found that bull and human sperm contain acetylcholine and choline acetyltransferase. The latter was five times higher in the tail of bull sperm than in the head or midpiece. Also, the tail appears to be higher in acetylcholine esterase than the head or midpiece. They proposed that the cholinergic system may play a role in

controlling sperm motility which is necessary for fertilization. In our studies, paraoxon, a known inhibitor of the cholinergic system, did not affect sperm motility but did inhibit fertilization. This inhibitory effect depends on pretreatment of sperm with paraoxon. Consequently, we propose that paraoxon inhibits fertilization of mouse gametes by affecting sperm capacitation. Once sperm are fully capacitated, paraoxon no longer affects fertilization. These effects of paraoxon may be in part attributed to paraoxon induced changes in elements of the cholinergic system, known to be present in sperm (Bishop *et al.*, 1976; Eusebi *et al.* 1978; Kusano 1977; Robbins and Molenaar 1981).

Table 4. Effects of treating mouse sperm with paraoxon (50 μM) for 1, 2, or 3 h prior to insemination on fertilization *in vitro*. In all cases sperm were incubated 3 h prior to insemination.

Treatment	Oocytes inseminated	Oocytes fertilized (%)[a]
Control	128	85(3)[b]
Paraoxon addition to sperm before insemination (h):		
0	99	65(13)[c]
1	84	45(8)[d]
2	94	51(21)[d]
3	95	42(12)[d]
3 (paraoxon diluted to 5 μM at insemination)	76	19(5)[e]

[a] Figures in parenthesis are standard errors.
[bcde] Means with different superscripts are different ($P < .01$).
[e] Different from control ($P < .001$).

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