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Aroclor 1254 Does Not Affect the IVF of Cumulus-Free Mouse Oocytes

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PCBs have been shown to produce many reproductive problems in many species. Oral doses of A-1254 in the female rhesus monkey, beginning 37 months prior to breeding, led to decreased conception rates, increased fetal mortality, and decreased infant head sizes (Arnold *et al.* 1995). PCBs decrease oocyte fertility *in vitro.* At levels of 0.1µg/ml and higher, A-1254 had adverse effects on mouse IVF (Kholkute *et al.* 1994a).

Following normal fertilization, and the subsequent cortical granule reaction (CR), enzymes released into the perivitelline space alter the zona pellucida (ZP) proteins, ZP3 and ZP2. These proteins are responsible for primary sperm binding and the induction of the acrosome reaction, and secondary sperm binding respectively, and are converted to ZP3, and ZP2, during this reaction. This creates a block to polyspermy. The zona reaction can also occur prematurely, during in *vitro* maturation in serum-free medium (Schroeder *et al.* 1988), or as a result of oocyte aging (Gianfortoni and Gulyas 1985). ZP "hardening", a term used to describe the result of the zona reaction, decreases the fertility of unfertilized oocytes and creates resistance to dissolution of the ZP by proteolytic enzymes such as α-chymotrypsin (DeFelici and Siracusa 1982).

PCBs effect various biochemical reactions in different cell types, which, if similar in the oocyte, could decrease fertility. Among these are the stimulation of the production of inositol phosphates in polymorphonuclear neutrophils, activation of protein kinase C, and alteration of calcium homeostasis in the rat cerebellar granule cells (Kodavanti *et al.* 1994).

The objective of the present research was to examine the functional effects of PCBs on mouse oocytes, using both a ZP dissolution assay and IVF. The hypotheses tested were that A-1254 both induces ZP hardening and causes decreased fertilization in cumulus-free mouse

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oocytes.

MATERIALS AND METHODS

Female C57BL6J and male DBA/2J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animals were housed in a 12 hour light:dark photoperiod at 23±2°C. All females were 8-12 weeks of age and males were mature at the time of experimentation.

A-1254, a mixture of PCB congeners, was purchased from Accustandard, Inc. (Lot #085-021, New Haven, CT) in neat form and dissolved in alcohol. It was diluted in culture media to final concentrations of 1.0 and 10.0 µg/ml, concentrations previously reported to produce adverse effects in a mouse IVF system (Kholkute *et al.* 1994a).

Females were injected i.p. with 10 I.U. pregnant mares serum gonadotropin (PMS, Sigma Chemical Co., St. Louis, MO). Forty eight hours later they were injected i.p. with 10 I.U. human chorionic gonadotropin (hCG, Sigma Chemical Co.). Cumulus-enclosed oocytes were collected 17-19 hours later.

Brinster's medium for oocyte culture with 0.5% BSA, (BMOC-3, Gibco BRL, Grand Island, NY) was used for all gamete collection and for IVF in Falcon organ tissue culture dishes (#3037, Becton-Dickinson and Co., Lincoln Park, NY). Media were prepared on the night before each trial and were equilibrated overnight in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C.

On the morning of the trial, one male was sacrificed by cervical dislocation. Two more males were sacrificed over the course of each experiment, 1-1.5 hours prior to use. The cauda epididymides were excised to BMOC-3, and punctured with a 25 gauge needle to release spermatozoa. The dish was returned to the incubator for 0.75-1.5 hours, to allow capacitation. Thirty minutes after sperm collection, 8-10 superovulated females were sacrificed. Cumulus masses were collected from the swollen oviducts. These were dispersed in 3.0 ml of PBS which contained 510 µg hyaluronidase/ml. The cumulus-free oocytes were rinsed and randomly distributed to the inner well of one of the seven experimental group dishes. The first group was the experimental control, in which oocytes were neither aged, nor exposed to PCB prior to their insemination with 50 to 75 µl of sperm suspension (2-3 X 10^6/ml). Three groups were incubated for three hours prior to insemination. These oocytes were exposed to either 0, 1.0 or 10.0 µg/ml A-1254 in BMOC-3. The other set of three was

identical except that the oocytes were incubated for six hours prior to insemination. Each group had between 8 and 37 oocytes.

The oocytes were assessed for fertilization 24 hr after insemination. An oocyte was considered fertilized if two polar bodies were present, or if it had developed to the 2-cell stage.

The protocol for zona hardening was identical (3 and 6 hour exposure trials). Cumulus masses were collected directly into Dulbecco's phosphate buffered saline (PBS, Gibco) at pH 7.8. The cumulus masses were then dispersed in 3.0 ml of PBS with hyaluronidase. These were rinsed and transferred to one of the three experimental groups (the 1.0 µg/ml A-1254 group, the 10.0 µg/ml A-1254 group, or the control group), or directly to a 200 µl droplet of 2 mg/ml α-chymotrypsin (Sigma Chemical Co.) in PBS. This final group, which was unaged, was the experimental control to determine if effects seen were due solely to aging. Following the 3 or 6 hour incubation period, oocytes were rinsed twice and transferred to 200 µl droplets (5-15 oocytes/droplet) of 2 mg/ml α-chymotrypsin in PBS.

Once transferred to α-chymotrypsin droplets, oocytes were assayed for the loss of their ZP every 5 minutes for the duration of the trial. Culture dishes were kept on a stage warmer at 37°C. The time required for 50% of the ZP to become solubilized, the $Lysis_{50}$ was used for comparison among groups.

The mean percentages of fertilization in the IVF trials were compared using ANOVA, and pairwise comparisons among groups were made using Tukey's test. The $Lysis_{50}$ of each group in the ZP hardening assay was compared using the Kruskal-Wallis t-sample test, which is a nonparametric rank-based test for use with data that are not normally distributed. For all tests $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

If PCBs act similarly in oocytes, as they do in other cell types, then one would expect to see a high degree of ZP hardening in PCB-exposed oocytes. As shown in Table 1 this was not the case. The values given are the mean $Lysis_{50}$ for 8 trials. Larger values indicate greater resistance to dissolution. Following 3 hours of incubation, A-1254 had no effect on ZP hardening. Hardening in the PCB groups did not differ from the aged control ($p>0.05$). Both the 10.0 µg/ml A-1254 and the aged control groups were significantly

Table 1.	Effects of A-1254 on zona pellucida hardening in cumulus-free mouse oocytes. (The numbers in parentheses signify the number of oocytes in the group).		
	$Lysis_{50}$ (min) Mean ± SEM		
Group	0 Hour	3 Hour	6 Hour
Control	27.1 ± 4.4 (322)	45.0 ± 7.4 (154)	45.0 ± 4.5 (130)
1.0 µg/ml A-1254		40.6 ± 5.0 (142)	24.4 ± 5.3 (148)
10.0 µg/ml A-1254		42.5 ± 5.4 (135)	26.3 ± 4.6 (148)
The Kruskal-Wallis test showed an overall significant difference between groups ($p<0.05$). Ohr control vs. 3 and 6hr controls, and 10.0 µg/ml A-1254 ($p<0.05$). 6hr 1.0 and 10.0 µg/ml A-254 vs. 3 and 6hr controls ($p<0.05$).			

different from the unaged control ($p<0.05$). Increased resistance to ZP dissolution was gained in the aged control group possibly due to limited cortical granule (CG) exocytosis, which occurs spontaneously during *in vitro* culture in denuded oocytes.

For the 6 hour groups the $Lysis_{50}$ for the aged control was the same as for the 3 hour group, suggesting that spontaneous CG exocytosis was completed by three hours of incubation. The $Lysis_{50}$ for the two A-1254 groups however, were reduced below the level of the unaged control, and were both significantly lower than that from the aged control ($p<0.05$). This was unexpected because the cortical reaction had already triggered ZP hardening by three hours of exposure to A-1254 in a nonreversible reaction.

A-1254 had no effect on ZP hardening following 1 hour of exposure time. The $Lysis_{50}$ for the 1.0 µg/ml A-1254 group did not differ from the unaged control, and that for the 10.0 µg/ml A-1254 was only slightly elevated. For the 1 hour aged control however, ZP hardening was evident. This demonstrates that spontaneous cortical granule exocytosis begins early during *in vitro* culture. The data also suggest that A-1254 slows the rate of spontaneous cortical granule exocytosis, as it does not begin until after one hour of culture.

Kholkute *et al.* (1994b) previously reported that A-1254 had adverse effects on IVF, using cumulus-enclosed oocytes aged for 6 hours prior to insemination. In contrast, we used cumulus-free oocytes. There were no differences in fertilization rates between groups ($p>0.8$; Table 2). Our data also demonstrates that the percentage of degenerated oocytes was elevated in the unaged control group. Degeneration in the 6 hour A-1254 groups was significantly lower than in the unaged group ($p<0.05$; Table 2).

Table 2. The effects of A-1254 on *in vitro* fertilization in cumulus-free mouse oocytes.

Group	Total Number of Oocytes	Number of Fertilized Oocytes (%)	Number of Degenerate Oocytes (%)
Control	141	70 (49.6)	23 (16.3)
3hr Control	136	64 (47.1)	12 (8.8)
3hr 1.0μg/ml A-1254	141	71 (50.4)	9 (6.4)
3hr 10.0μg/ml A-1254	141	68 (48.2)	15 (10.6)
6hr Control	144	78 (54.2)	8 (5.6)
6hr 1.0μg/ml A-1254	125	71 (57.0)	3 (2.4)
6hr 10.0μg/ml A-1254	150	61 (40.7)	6 (4.0)

ANOVA showed no significant difference in fertilization rates between groups. The percentage of degenerate oocytes in the control group was significantly greater than the 6hr A-1254 groups ($p<0.05$).

In the present studies, fertilization was not affected at either 1.0 or 10.0 µg/ml of A-1254, nor was it affected by duration of incubation. The percentage of degenerate oocytes was also unaffected by A-1254.

The results suggest that A-1254 had no direct influence on ZP hardening. At 1 hour, the $Lysis_{50}$ for the PCB groups was not elevated, while for the aged control it was. Spontaneous CG exocytosis did not occur in the PCB groups by three hours. This increase occurred to a lesser extent than in the aged controls.

The decrease in ZP hardening in the A-1254 groups at 6 hours, to below that for the unaged control, gives further evidence that the toxin does not directly affect CG exocytosis. The addition of β-mercaptoethanol, a reducing agent, to serum-free medium during *in vitro* oocyte maturation, prevents ZP hardening (Zhang *et al,* 1991). This suggests that ZP hardening results from protein cross-linking by disulfide bonds. Cross-linking of the glycoproteins, which undergo limited proteolytic cleavage during the zona reaction (Wasserman and Mortillo 1991) results in conformational changes of the ZP such that ZP1 becomes shielded from the extracellular environment. It is also preferentially cleaved by α-chymotrypsin, resulting in the loss of interconnections between filaments and dissolution of the ZP. It would appear that over time in culture, A-1254, could alter the conformation of the modified ZP, making ZP1 more available to α-chymotrypsin, and thus decrease the resistance of the ZP to dissolution.

Another potential reason why PCBs did not induce CG exocytosis may be a result of their effects on calcium homeostasis. PCBs have been shown to induce large and sustained increases in intracellular calcium

levels. Following gamete fusion in mouse oocytes, transient oscillations in intracellular calcium levels begin, and following the first two transients, continue to occur at twenty minute intervals for up to 4 hours (Cuthbertson and Cobbold 1985). These transient calcium oscillations are essential for the initiation of activation events, including meiotic resumption, and the extrusion of the second polar body (Kline and Kline 1992).

The results from both IVF and the ZP hardening assays suggest a role for the cumulus cells in PCB-induced toxicity in the oocyte. A-1254 neither stimulated nor inhibited CG exocytosis, nor did it lower IVF in cumulus-free oocytes.

The regulation of PKC represents another potential site for cumulus cell mediated effects. A 74-kDa phosphorylated protein was present only in granulosa cell-enclosed oocytes (Wong and Pessah 1996). This suggested that a signal was sent to the oocyte from the granulosa cells, and that this signal regulated protein phosphorylation by PKC. It is possible that PCBs could block this signal, thereby inhibiting PKC activity, and CG exocytosis, and could potentially cause polyploidy, as the slow block to polyspermy would not be created. This could explain results of Kholkute *et al.* (1994b) who found that A-1254 caused an increase in the percentage of degenerate oocytes.

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