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Effects of Polychlorinated Biphenyl (PCB) Mixtures on *In Vitro* Fertilization in the Mouse

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Polychlorinated biphenyls (PCBs) are persistent environmental contaminants that have been reported to adversely affect reproduction in both laboratory and wild mammals (Jonsson *et al.* 1976; Barsotti *et al.* 1976; Orberg 1978; Bleavins *et al.* 1980). However the effects of these industrial chemicals on oocytes, oocyte-sperm interaction, and fertilization are lacking. Our recent study demonstrated adverse individual effects of Aroclor (A)-1221, A-1254, and A-1268 and the PCB congener 3,3',4,4'-tetrachlorobiphenyl on *in vitro* fertilization (IVF) in the laboratory mouse (Kholkute *et al.* 1994a,b). Mixtures of PCBs were not tested. Each of these chemicals caused an increased incidence of degenerative oocytes and abnormal 2-cell embryos *in vitro.* The fact that the environment is contaminated with mixtures rather than individual, PCBs raises the issue of whether or not one PCB can influence the biologic and/or toxic effects of another PCB. In the present study we have evaluated the *in vitro* effects of two commercial PCB mixtures on fertilization and early embryogenesis in the mouse using IVF as a test system.

MATERIALS AND METHODS

Male DBA/2J and female C57BL/6J mice were used. These mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and aged 2-3 months and 7-9 weeks, respectively. The B6D2Fl hybrids were produced by mating DBA/2J males with C57BL/6J females at the Endocrine Research Center of Michigan State University. All animals were housed in Plexiglas boxes (4 mice per box) under a 12 hour light/dark photoperiod and maintained in an air conditioned room at 24 ± 2°C. Feed (Mouse Chow® #5015, Purina Mills, Inc.) and water were available *ad libitum.*

Brinster's medium (Brinster 1971) for oocyte culture with 0.4% bovine serum albumin (BMOC-3, GIBCO, Grand Island, NY) was used for incubation of oocytes (15 to 30 per well) and sperm in the center wells of Falcon organ tissue culture dishes (Becton-Dickinson and Co., #3037, Cockeysville, MD). A similar medium without BSA (BMOC) was used in the outer wells of tissue culture dishes. Commercial Aroclor mix-1

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(containing Aroclor 1016, 1232, 1248, and 1260) and Aroclor mix-2 (containing Aroclor-1221, 1242, and 1254) were purchased from AccuStandard, Inc. (New Haven, CT) and dissolved in ethyl alcohol. Individual solutions of the Aroclor mixtures were then suspended in BMOC-3 to obtain 0.1, 1.0 and 10.0 µg/ml concentrations with a maximal alcohol content of 0.01% (V/V). Culture dishes were loaded with 1 ml of medium containing the various concentrations of either mix-1, mix-2, or control medium. BMOC-3 (1 ml) was added to the center well for collection of spermatozoa. Organ culture dishes were similarly prepared for collection of cumulus masses containing the oocytes. BMOC (3 ml) was placed in the outer wells of all culture dishes. The culture dishes were equilibrated overnight in a humidified incubator at 5% CO_2 + 95% air at 37°C.

Female mice (4-6 weeks old) were superovulated by injecting (ip) 10 IU pregnant mare's serum gonadotropin followed by 10 IU human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO) 46-48 hrs later. Twelve to 15 hrs following hCG, 2 adult male mice (3 to 5 months old) were sacrificed by cervical dislocation. The cauda epididymides of each male were excised and placed in the center well of the organ culture dish containing 1 ml BMOC-3. They were repeatedly punctured with a 25 ga needle to release the sperm. The sperm suspension thus obtained was incubated for 1.5 hr. Sperm motility estimates were 60% or greater for each trial.

Approximately 45 minutes after sperm collection, 5 to 7 superovulated females were sacrificed by cervical dislocation. The ovaries and oviducts with part of the uteri were excised and kept in the center well of the culture dish. Cumulus masses were recovered from the dilated ampullae under a dissecting microscope, washed once in BMOC-3 and transferred to the center wells of control or treatment organ culture dishes. The cumulus masses were randomly placed across replicates to avoid the same female donating to the same group. Fifty µl of sperm suspension was then added directly to the center well. Each replicate was inseminated by a different male. The dishes were then placed in the incubator for an additional 20-24 hrs.

Prior to IVF assessment, vital nuclear stain (0.002g) Bisbenzimide H 33258 fluorochrome trihydrochloride pentahydrate (Calbiochem-Benning, La Jolla, CA) was placed in 10 ml of BMOC. From this, 0.1 was added to each culture dish and then replaced in the incubator for a 30 minute period. At the end of the incubation period, each culture dish was scored for the percentage of oocytes fertilized. Oocytes were considered fertilized by the presence of two cells, two cells with one polar body or one cell with two pronuclei. Oocytes were considered unfertilized by the presence of one cell with one pronucleus or if degenerative. The

degenerative oocytes were characterized either by compact contracted dense ooplasm or by fragmented appearance. The abnormal 2-cell embryos showed fragmentation or irregular cell mass. The number of degenerative oocytes and abnormal 2-cell embryos were recorded. The cultures were observed on a Nikon Diaphot inverted microscope equipped with a fluorescent objective (40X) using a Nikon epifluorescence filter set (365/10 exciter, 400 dichroic mirror, 400 barrier filter) at 365-nm wave lengths with a mercury arc lamp.

Results of the IVF trials were analyzed by Chi Square and Bonferroni Chi Square contingency tables (Gill 1987). Individual cultures (wells) represented the experimental unit. The differences between groups were analyzed by individual Chi Square test.

RESULTS AND DISCUSSION

Exposure to Aroclor mixes resulted in a highly significant effect on the percent fertilization, abnormal embryos and degenerative oocytes (mix-1: $X^2=61.3$, $p<0.001$, d.f.=1; mix-2: $X^2=65.03$, $p<0.001$, d.f.=1; Table 1). We observed a significant ($p<0.05$) effect on all the parameters at 1 and 10 µg concentrations compared to the control and the 0.1 µg treatment groups. However, there was no significant difference ($p > 0.05$) between the 1 and the 10 µg treatment groups.

The results of the present *in vitro* study demonstrated adverse effects of Aroclor mix-1 and Aroclor mix-2 on fertilization, oocytes, and 2-cell embryos in the mouse. The mechanism of action of PCBs in effecting fertilization is not known. However, a possible mechanism may be the disruption of Ca^{++} homeostasis which is critical for capacitation, gamete interaction, and fertilization (Yanagimachi 1981). In rat cerebellar granule cells, it was shown that PCBs alter intracellular calcium homeostasis (Kodavanti *et al.* 1993). Following ten minutes of exposure to PCBs, intracellular calcium levels were increased, and it was found that calcium uptake by mitochondria and other organelles was inhibited, as was synaptosomal Ca^{2+}-ATPase which is involved in calcium extrusion from the cytoplasm. This resulted in a sustained rise, rather than a natural transient rise, in intracellular calcium concentrations due to the inhibition of the cellular buffering systems. A rise in intracellular calcium initiates a second messenger cascade, culminating with the activation of protein kinase C (PKC). Activation of PKC has been seen following PCB exposure in rat cerebellar granule cells (Kodavanti *et al.* 1994; Kodavanti *et al.* 1995), as well as in brain extracts from both rats and mice (Shukla and Albro 1987). In the oocyte, PKC acts as a mediator of calcium-induced CG exocytosis, by phosphorylating cellular substrates that are involved in the regulation of exocytosis (Bement 1992). PCBs may also perturb the

Table 1. Effects of Aroclor mixtures on mouse *in vitro* fertilization.

Group	Total Ova	No. Fertilized (%)	No. Abnormal Embryos (%)	No. Degenerating Ova (%)
Aroclor Mix-1				
Control	144	127 (88.0)	3 (2.1)	4 (2.7)
0.1 μg	134	69 (51.5)	7 (5.2)	10 (7.4)
1.0 μg	170	70 (41.1)[a]	11 (7.0)[a]	25 (14.7)[a]
10.0 μg	180	61 (34.0)[a,b]	17 (9.4)[a,b]	17 (9.4)[a,b]
Aroclor Mix-2				
Control	136	117 (86.0)	2 (1.4)	4 (2.0)
0.1 μg	130	72 (55.2)	6 (4.6)	12 (9.2)
1.0 μg	154	61 (39.6)[a]	12 (7.8)[a]	24 (15.6)[a]
10.0 μg	151	50 (33.1)[a,b]	18 (11.9)[a,b]	32 (21.2)[a,]

Individual Chi Square

[a] $p < 0.05$ compared to control and 0.1 µg groups (tested separately)

[b] $p > 0.05$ 1 µg vs 10 µg not significantly different from 1 µg level.

membrane integrity of the gametes, thereby affecting fertilization. Both Aroclor mixtures showed a direct deleterious effect on oocytes, an essential cellular element in the ovary and on early embryogenesis. Our earlier study (Kholkute *et al.* 1994a,b) with Aroclor-1221, 1254, 1268, and 3,3',4,4'-tetrachlorobiphenyl individually, demonstrated adverse effects on fertilization and increased incidence of degenerative oocytes and abnormal embryos just as was observed for Aroclor mixtures in the present studies. A-1221 and A-1254 at 10 µg/ml concentration reduced the IVF rate to 34% and 37% respectively, values comparable to the two Aroclor mixtures in the present study. The mixes in this study had no apparent additive or synergistic effect as there was no further enhancement of activity compared to A-1221 and A-1254 when used separately. The similar response of commercial mixtures 1 and 2 (They were not significantly different) suggests that one or more of the Aroclors in mixture 1 will adversely affect fertilization at the higher levels if tested separately.

In conclusion, the present *in vitro* study demonstrates adverse effects of Aroclor mixtures on oocytes, fertilization and early post-fertilization events in the mouse without additive or synergistic effects.

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