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Aroclor-1260® Effects on *In Vitro* Fertilization in the Mouse

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Polychlorinated biphenyls (PCBs) are persistent industrial chemicals of global environmental concern. PCB residues have been detected in fish and wildlife as well as in human adipose tissue, blood, and milk (Mes et al. 1982 ; Schwartz et al. 1983; Swain 1988; Yukushiji 1988). The effects of PCBs on reproduction were first studied in the mink (Ringer et al. 1972). Since then the reproductive toxicity of many commercial and PCB congeners have been reported. Our earlier studies demonstrated adverse individual effects of Aroclor (A)-1254, 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). We reported adverse effects of an Aroclor mixture that contained A-1260 (Kholkute and Dukelow 1997) but A-1260 had not been tested independently.

Our earlier experiments concentrated on freshly-collected oocytes. The objectives of the present study were to evaluate the effects of A-1260 on IVF of frozen-thawed and fresh oocytes. Four concentrations (0.01, 0.1, 1.0, 10.0 µg/ml of A-1254 and A-1268 were tested in our earlier studies (Kholkute et al. 1994a,b) but 0.01 µg/ml PCB did not have significant effects on IVF in the mouse oocytes. Higher levels showed effects. Therefore, only three concentrations (0.1, 1.0, 10.0 µg/ml) were chosen for testing in this study.

MATERIALS AND METHODS

Male B6D2F1 and female ICR (CD-1) mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) at age 2-3 months and 4-6 weeks, respectively. All mice were housed individually in plexiglass boxes under a 12-hour light/dark photoperiod and maintained in an air conditioned room at 23±2°C. Feed (Mouse Chow® #5015, Purina Mills, Inc.) and water were available ad libitum.

Brinster's medium (Brinster, 1971) for oocyte culture

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with 0.4% bovine serum albumin(BMOC-3, GIBCO, Grand Island, NY) was used for incubation of oocytes (15-30 per well) and sperm in the center wells of Falcon organ tissue culture dishes (#3037, Becton-Dickinson and Co., Franklin Lakes, NJ). A similar medium without BSA (BMOC) was used in the outer wells of tissue culture dishes for humidity control.

Aroclor-1260, 99% pure as determined by gas chromatography and a flame ionization detector, was purchased in neat form from AccuStandard, Inc. (New Haven, CT), dissolved in ethyl alcohol, and serially diluted in culture medium to obtain the desired concentrations with the maximal alcohol content of 0.01% (v/v). Three concentrations of A-1260 (0.1, 1.0, 10.0 µg/ml) were studied with freshly collected oocytes. Only two concentrations of A-1260 were tested with frozen oocytes. The frozen oocyte trials were conducted first and because of the increased oocyte degeneration in that environment, trials were curtailed and greater emphasis placed on the fresh oocyte trials. Control culture dishes contained culture medium with 0.01% (v/v) alcohol. For oocyte fertilization trials, culture dishes were loaded in the center wells with 1 ml of BMOC-3 containing the various concentrations of A-1260. The outer wells had 3 ml BMOC for humidity control. For collection of spermatozoa, one ml of BMOC-3 was added to the center well. A sterilized 4-well petri dish was used for collection of cumulus masses containing oocytes. Two ml of BMOC-3 was added to each of the 4 wells. Both the culture dishes and the 4-well petri 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 intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin followed by 10 IU human chorionic gonadotropin (hCG) 46-48 hrs later. Twelve to 15 hrs following hCG injection, 2 adult male mice (3-5 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 without A-1260. They were then repeatedly punctured with a 25 gauge needle to release the sperm. The sperm suspension thus obtained was incubated for 1.5 hr for capacitation. Sperm motility was assessed 30 minutes after incubation and samples showing >60% motility were used for insemination.

Approximately 45 minutes after sperm collection, 5 to 7 superovulated female mice were sacrificed by cervical dislocation. The ovaries and oviducts and a part of the uteri were excised and kept in one of the 4-well petri dishes, washed once and transferred to another well. Cumulus masses were recovered from the dilated ampullae

under a dissecting microscope, washed once in BMOC-3 and directly transferred to the center wells of control or treatment culture dishes, or to a well to be processed for cryopreservation. The cumulus masses were randomly placed among replicates to avoid the same female donating to the same group. Fifty µl of sperm suspension (2-3 x 10^6 sperm) was then added directly to the center well. The dishes were then promptly returned to the incubator for an additional 20-24 hrs.

For cryopreservation, cumulus masses were washed in BMOC-3 containing 200 µg/ml hyaluronidase for 3 to 5 minutes. Oocytes were then further washed in BMOC-3 and 1.5 M glycerol in PBS. Straws (0.25 ml) were prepared for oocytes by loading a 7.5-cm column of 1.0 M sucrose in PBS followed by a 0.5 cm column of air and a 1.0 cm column of 1.5 M glycerol solution, respectively. After five minutes incubation of oocytes in the cryoprotectant, the straws were loaded with 15 to 30 oocytes into the glycerol solution of each straw and heat-sealed. Straws containing oocytes were placed into a programmed BioCool-III (FTS Systems, Stone Ridge, NY) holding at -7°C for 10 minutes, then cooled at 0.5°/minute from -7 to -40°C. The straws were held at -40°C for 10 minutes, then plunged into liquid nitrogen.

Cryopreserved oocytes were thawed by placing straws in air at room temperature for two minutes, then three minutes in water at 37°C, and then two minutes in room temperature water. Oocytes were expelled from the straws into BMOC-3 and washed twice in BMOC-3 for use with in vitro fertilization trials.

At the end of the incubation period, each culture dish was examined under a dissecting microscope and 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 (Kholkute et al, 1994a). Oocytes were considered unfertilized by the presence of one cell with one pronucleus or if degenerative. The number of degenerative oocytes was recorded. These were characterized by a compact, contracted dense ooplasm or fragmentation.

Data from the IVF trials (% fertilized and % degenerative oocytes) were analyzed by Chi-square test.

RESULTS AND DISCUSSION

Chi-square analysis of the percentage of oocytes fertilized showed no significant differences between freshly-collected and frozen-thawed oocyte controls. However, there were significant effects ($p<0.05$) of all

the A-1260-treated groups compared to respective controls (Table 1).

Table 1. Effects of A-1260 on IVF in the mouse

Group	Total oocytes	No. fertilized Oocytes (%)
Fresh oocytes		
Control	196	142 (72.4)
0.1 μg/ml	199	123 (61.8)[a]
1.0 μg/ml	310	159 (50.7)[a,b]
10.0 μg/ml	188	82 (43.6)[a,c]
Frozen oocytes		
Control	51	36 (70.6)
1.0 μg/ml	54	27 (50.0)[a]
10.0 μg/ml	43	17 (39.5)[a,c]

Chi-square analysis of % fertilized oocytes showed no significant differences ($P < 0.05$), between freshly-collected and frozen-thawed oocyte controls, but A-1260 did have significant effects ($p>0.05$) on % fertilized oocytes of both groups.

[a] $p<0.05$ vs. control

[b] $p<0.05$ vs. 0.1 μg/ml

[c] $p<0.05$ vs. 1.0 μg/ml

Analysis of percentage of degenerate oocytes revealed that there were significant differences ($p<0.05$) in oocyte degeneration in IVF between fresh and frozen oocyte controls, however, A-1260 did not exhibit degenerative effects ($p>0.05$) on IVF in all the PCB-treated groups (Table 2).

Consistent with our earlier studies on A-1254 and A-1268, A-1260 at 20.1 μg/ml decreased the IVF rate, but had no effect on oocyte degeneration. Although there was an increased incidence of degenerative oocytes in the frozen-thawed oocyte group, Chi-square analysis demonstrated that these differences were due to exposure of oocytes to the frozen-thaw process instead of to A-1260. The mechanisms of action of A-1260 and other PCBs in reducing IVF is not known; however, it is possible that it may affect membrane permeability and/or disrupt Ca^{2+} homeostasis, an important event in sperm-egg interactions and fertilization (Yanagimachi 1981). Polychlorinated biphenyls have been detected in human follicular fluid (Trapp et al. 1984) and human ovarian tissues (Mes 1990). Although the concentrations of PCBs reported in follicular fluid (4.7-27 ppb) is far lower

Table 2. Effects of A-1260 on oocyte degeneration in the mouse

Group	Total Oocytes	No. degenerate oocytes (%)
Fresh oocytes		
Control	196	8 (4.1)
0.1 μg/ml	199	14 (7.0)[a]
1.0 μg/ml	310	30 (9.6)[a]
10.0 μg/ml	188	17 (9.0)[a]
Frozen oocytes		
Control	51	6 (11.8)
1.0 μg/ml	54	13 (24.1)[a]
10.0 μg/ml	43	9 (21.9)[a]

Test of % degenerate oocytes by Chi-square revealed a significant difference ($p<0.05$) between freshly-collected and frozen-thawed oocyte controls, but A-1260 did not have significant effects ($p<0.05$)on the percentage of oocyte degeneration.
[a] $p>0.05$ vs. control

than the concentrations of PCBs that adversely affected the mouse IVF rate (1.0 µg/ml, i.e., 1 ppm), the results suggest that a high accumulation of these environmental pollutants may prove detrimental to the fertilizing ability of the oocytes.

It is reported that intercellular communication between the oocyte and granulosa cells by gap junctions is necessary for oocyte growth in vitro(Bacharova et al. 1980, Herland and Schultz 1984). Polychlorinated biphenyls have been shown to inhibit gap junctional communications in cultured liver cells (Swierenga et al. 1990), and this may be one of the mechanisms causing oocyte degeneration. They have also been shown to be cytotoxic in Chinese hamster ovary (CHO-KI) cells in vitro (Rogers et al. 1983) and affect cell viability in mouse Leydig cells (Johansson 1989). Although it is inconclusive to extrapolate the results of the present in vitro study with in vivo studies, the increased incidence of abortion in rhesus monkeys following long-term exposure to polybrominated biphenyl (Lambrecht et al. 1978) could be due to oocyte toxicity, fertilization failure and early embryo effects similar to those seen in the present study.

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