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THE EFFECT OF AROCLOR-1254 ON THE DEVELOPMENT OF 8 CELL MOUSE EMBRYOS

By

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ABSTRACT

THE EFFECT OF AROCLOR-1254® ON THE DEVELOPMENT OF 8-CELL MOUSE EMBRYOS.

By

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Polychlorinated biphenyls (PCBs) accumulate in the environment from past human activities, and continue to disperse globally even though their applications are restricted . In the United States, Aroclor-1254, a commercial PCB mixture was among the most widely used and more toxic than other higher chlorinated Aroclors. Exposure to PCBs has caused adverse effects on reproduction in a wide range of laboratory animals. For this investigation three different concentrations of Aroclor-1254 (0.1, 1.0, and 10 µl/mL) were tested. Eight cell mouse embryos were recovered from superovulated C57BL/6J females at approximately 57 hours. Observations were made every 24 hours after collection for up to 96 hours. The results indicated a significant effect ($P < 0.05$) at the three concentrations of Aroclor-1254 (A-1254), on the development of the 8-cell mouse embryo, at 48 hours during the morula stage. Also, a significant effect was obtained at 72 hours ($P < 0.05$) at the 1.0 µl/mL concentration of A-1254.

To my wife Brenda, my parents, and to my brothers and sisters in Puerto Rico for their unconditional, love, support and understanding now and always.

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INTRODUCTION

Polychlorinated biphenyls (PCBs) were first synthesized in 1881 and commercial production began in the United States in 1929. Since their first commercial production, an estimated 1.2 million tons of PCBs have been produced worldwide (Tanabe, 1988). The properties of improved chemical stability, low solubility in water, low flammability, and excellent insulating properties, provide many commercial benefits for the use of PCBs as heat transfer fluids (e.g., in electrical transformers and capacitators), flame retardants, lubricating and hydraulic oils, additives in plastic, and innumerable other applications (Pomerantz et al., 1978). These extensive uses, together with their physical/chemical properties, led to their extensive distribution in the environment in the late 1960s.

Due to the widespread occurrence of PCBs in the ecosystem and their potential adverse effects on animals, including humans, significant efforts have been invested by government and industry throughout the world to understand how PCBs function and what possible hazard could occur from their presence in the environment.

Reproduction is considered one of the most sensitive methods of testing compound toxicity. Maternal consumption of PCB contaminated fish in humans has been reported to cause reduced birth weight, diminished head circumference, and reduced gestational age (Fein et al., 1984 ; Swain, 1991).

Exposure to PCBs has caused adverse effects on reproduction in a wide range of laboratory animals. In rodents, these effects have included embryo and fetal toxicity, decreased fertility in both males and females, and decreased survival of offspring (EPA,

al., 1987; Golub et al., 1991). Previous studies in this laboratory (Kholkute et al., 1994a) demonstrated that Aroclor-1221, 1254 and 1268 adversely affected in vitro fertilization in mice, Aroclor-1254 being the most toxic. The primary objective of this study was to investigate the effects of Aroclor -1254 on the development of the 8-cell mouse embryo.

LITERATURE REVIEW

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are among the most extensive and constant environmental contaminants (Swain, 1983). These industrial chemicals were marketed under trade names such as Aroclor, Clophen, and Phenclor. They were first described and synthesized in 1881, with commercial production and application beginning in 1929. The world's major producer of PCBs was The Monsanto Company. They stopped production of PCBs in 1971, but continued production for closed uses (e.g.; closed heat-transfer systems, electrical transformers, condensers). In 1977, Monsanto stopped production completely. Since then the sale and use of these chemicals in the United States have been strictly controlled by federal law.

Commercial PCB products were mixtures of up to 209 congeners, with different physical and chemical characteristics. In the United States, the major registered trade name for commercial PCB mixtures was Aroclor. The name Aroclor was followed by a four digit number. The first two digits referred to the carbon atoms of the biphenyl ring and the last two digits referred to the percent chlorine by weight. Aroclor-1254 was among the most widely used and more toxic than other higher chlorinated Aroclors, such as A-1260, A-1262, and A-1268 (Kimbrough et al., 1978) .

PCBs are colorless, liquids or solids which are inflammable, electrically insulating, and chemically stable, which accounts for their persistence in the environment. These characteristics also made PCBs resistant to acid-base reactions, hydrolysis, chemical

oxidation, photodegradation and thermal changes, in addition to being extremely soluble in oils and fat. Because of this, they tend to partition out of the aquatic ecosystem into biological tissue.

Some of the uses for PCBs include; electrical transformers, capacitators, heat transfer plasticizers, paints, printing inks, suspension vehicle for the pigment in carbonless copy paper, sealants, adhesives, flame retardants, microscope immersion oil, and lubricants.

Human exposure to PCBs in the environment is widespread because these persistent chemical mixtures have entered the food chain. Occupational and accidental exposures have occurred due to the use of these chemicals in a variety of product applications and manufacturing processes (Swanson et al., 1995). Food has been shown to be the most important route of exposure for the general population, and milk products have been shown to contribute significantly to the total exposure (Krokos et al., 1996).

PCBs were first detected in milk in 1970 (Willett & Hess, 1975). The principal source of residues was farm silos in the eastern United States that had been coated with a PCB-containing coating (Krokos et al., 1996; Willett, 1983). Similarly treated silos have also resulted in milk contamination in Switzerland (DeAlencastro et al., 1984). Since human milk is part of the food chain, breast-fed infants will consume the highest concentration of persistent chemicals accumulating in the environment (Drijver, et al., 1988). PCBs in human milk fat are correlated to the corresponding levels in depot fat. Thus, supporting the view that comparisons of the levels of PCB contaminants in human milk should be based on the milk fat content (Koidu, 1983).

In Michigan, predominantly an industrial state, environmental contamination by PCBs as well as other toxic substances, has been a widely reported problem (Wickizer et al.,

1981). Reports of elevated levels of PCBs in fish in the Great Lakes (Willford et al., 1976), lead to controls being placed on commercial fishing and to recommendations by public health officials that persons reduce or eliminate their consumption of such fish.

Developmental effects attributed to PCB exposure include low birth weight and retarded growth in mice (McCoy et al., 1995). Prenatal exposure to PCBs has been linked to teratological effects such as cleft palate in the mouse and pig (Fuller & Hobson, 1986). Adverse human reproductive and developmental deficits have been linked to PCB exposure from consumption of contaminated fish or by direct agricultural contact (Hansen, 1994).

Subacute or chronic exposures to PCBs pre- or postnatally, pre- or postpubertally, as adults, or during pregnancy, have been found to be detrimental to reproductive functions in laboratory and wild mammalian species (Fuller & Hobson, 1986). Measurable concentrations have been detected in human ovarian tissue (Mes, 1990) and human embryos and fetuses (Nishimura et al., 1977).

Chemically induced reproductive disorders have been documented in different mammalian species including humans. Some of these effects have been attributed to PCBs (Lindenau & Fischer, 1996). In mice and rats, PCBs have been reported to affect the estrous cycle (Orberg & Kihlstrom, 1973) and uterotrophic activity (Gellert, 1978).

Embryo transfer experiments showed that morulae exposed to 5μg Aroclor-1260 had clear signs of degeneration after 24 hours in vitro culture but were able to implant (Lindenau & Fischer, 1996). One of the primary concerns, regarding PCB exposure in mammals, is the deleterious effects on many phases of reproduction in both sexes (George

& George, 1990). Studies of in vitro fertilization in mice indicated detrimental effects of PCBs on oocytes, fertilization, and early cleavage (Kholkute et al., 1994).

Mouse Development

The mouse is well established as an experimental mammal for developmental and genetic studies (Hennig, 1992). Mouse embryonic development has been studied in detail and has been well described (Theiler, 1989; Rugh, 1990).

The estrous cycle is 4-5 days and the gestation period is 19-20 days, depending on the strain. Mouse lifespan in the laboratory is 1.5-2.5 years. The average litter size is approximately 6-8 pups. In order to maintain regular cycling, a day-night photoperiod should be kept constant. Under this cycle females tend to ovulate once every four to five days, 3-5 hours after the onset of the dark period (Hogan et al., 1996).

Males under the same lighting conditions will copulate with females in estrus. Ovulation and fertilization are assumed to occur about 2:00 a.m., under our photoperiod at about the mid-point of the dark period. The morning after mating, the females can be checked for the presence of a copulation plug in the vagina (vaginal plug) as an indication of mating. This consists of coagulated proteins from the male seminal fluid and can easily be seen.

Males reach maturity at 6-8 weeks of age depending on the strain. Once used for mating, females they should be kept in individual cages, or they will fight. The sexual maturity of females is a major factor affecting the number of oocytes that are ovulated. The best age for superovulation usually falls between 3 and 5 weeks. Age, however, is not always a reliable indicator of the sexual maturity of the female; the nutritional status and health of a female mouse can also affect follicular maturation (Hogan et al. , 1986).

Pre-Implantation Mouse Embryo Development

Fertilization, the fusion of the female and male gametes, activates the oocyte to enter the complex process of embryogenesis. Preimplantation development takes about 4-5 days in the mouse. Cleavage (cell division) occurs while the cells are still surrounded by the zona pellucida, resulting in the production of many smaller cells, the blastomeres.

Cleavage takes place much more slowly in mammals than in most lower vertebrates or invertebrates. Frog eggs cleave about once an hour, and goldfish eggs every 20 minutes ; but a mouse egg takes 24 hours for its first cleavage division, and 10-12 hours for each succeeding division. (Austin & Short, 1982). Table 1 (Rugh, 1990) indicates the time and stage for the development of the pre-implantation mouse embryo.

During the first two days of development, blastomeres are spherical cells which are loosely attached at their sites of contact. At the eight cell stage blastomeres alter their adhesive behavior and the embryo undergoes a profound morphological change. The cells flatten towards each other, maximize cell-cell contacts and the former grape-like structure is transformed into a compact aggregate of cells. This phenomenon is called compaction and is dependent on the presence of calcium (Whitten, 1971) One major component of this process is uvomorulin (UM) , a trans-membrane glycoprotein which is also known as E-cadherin (Hyafil et al., 1980; Vestweber & Kemler, 1984; Yoshida-Noro et al., 1984). Uvomorulin is involved in Ca^{2+} dependent cell-cell adhesion not only in preimplantation embryos, but also in many epithelial cells later during development in adult tissues.

Table 1: Estimated early cleavage time table. [1]

Stage of Development	Time Required (Hours)
Coitus	0
First cleavage spindle	21-28
Two cell	21-43
Four cell	38-50
Eight cell	50-64
Sixteen cell morula	60-70
Blastocyst	66-82
Transport to uterus	66-72
Implantation	4-5 days

[1] From Rugh, 1990.

After compaction, fluid accumulates between intercellular spaces and around the 32-cell stage the blastocoel (cavity of the blastula) becomes evident. Outer cells pump fluid into the blastocoel which rapidly expands. Two distinct cell populations are present in the blastocyts: an outer layer of trophectodermal cells which represents a true epithelium surrounding the blastocoel, and the inner cell mass (ICM) cells, a group of cells which is attached to one side of the inner surface of the trophectoderm (Hogan et al., 1986).

The trophectoderm and inner cell mass cells remain totally distinct lineages from the onset of cavitation (blastocyst formation) (Dyce et al., 1987). The trophectodermal cells give rise exclusively to extraembryonic tissue. Shortly before implantation some of the inner cell mass cells differentiate into a second epithelial cell type, the primitive endoderm, which arises on the free surface of the ICM facing the blastocoel. The remaining ICM will give rise to the embryo proper and to the extraembryonic mesoderm (Austin & Short 1982).

Implantation occurs on day 4-5 of development. Before the blastocyst can be implanted it has to escape from its zona pellucida. This process is called hatching and is brought about by localized proteolysis of the zona pellucida and contraction and expansion of the blastocyst. A small hole is lysed into the protein matrix of the zona pellucida by a trypsin-like protease. The blastocyst hatches through this hole and once freed from its zona pellucida, attaches to the epithelium of the lateral uterine wall opposite from the ICM. A detailed analysis of the orientation of mouse embryos during implantation and a discussion of how this might be related to embryonic axis formation is given by Smith (1980, 1985).

Development of the 8-cell mouse embryo

The 8-cell stage of mouse development is characterized by a number of morphological changes that are described under the general heading of compaction (Johnson & Maro, 1984). Whereas in the early 8-cell stage individual blastomeres are rounded, during compaction the cells flatten against one another to maximize cell contacts, the intercellular boundaries becoming indistinct at the level of the optical microscope (Lewis & Wright, 1935; Lehtonen, 1980).

At the same time, the cells develop an apical "epithelial-like" localization of microvilli (Callarco & Epstein, 1973; Ducibella et al., 1977; Reeve & Ziomek, 1981). They develop specialized cellular junctions (Ducibella & Anderson, 1975; Magnuson et al., 1978; Lo & Gilula, 1979; Goodall & Johnson, 1984) and show a redistribution of intracellular organelles (Ducibella, et al., 1977; Reeve, 1981; Reeve & Kelly, 1983).

During compaction of the 8-cell embryo, the microtubules play a constraining role, being involved in the regulation of change in cell shape and organization and in their timing, rather than being actively involved in the actual mechanism of cell flattening and polarization (Maro & Pickering, 1984).

According to Hogan et al., (1986), one of the essential features of compaction is the polarization of the blastomeres, so that they show distinct apical and basolateral membrane domains. These domains are clearly seen by scanning electron microscopy of compacted embryos. Polarity-inducing ability arises at the two cell stage and increases up to the 8 to 16 cell stage.

Bavister (1987) reported that the permanent cellular polarity established at the 8-cell stage may be crucial for successful embryonic development since it provides the

foundation for the formation of the two distinct cell lineages leading to the trophectoderm and inner cell mass of the blastocyst.

MATERIALS AND METHODS

Animals

Female C57BL/6J and male DBA/2J mice both eight weeks old were used for the production of 8-cell embryos. They were purchased from The Jackson Laboratory (Bar Harbor, ME).

Mice were housed in Plexiglass boxes 7" x 11 ½" x 5" (Allentown Caging, Allentown, NJ) containing Bed-o-cobs® (Anderson Co., Maumee, OH). They were provided Teklad Rodent diet® (Madison, WI) and water ad libitum. All animals were kept at 12 hours light : dark photoperiod and maintained in an air conditioned room at 23 ± 2° C. The housing, maintenance and care conditions were in accord with state and federal regulations.

Aroclor 1254

Aroclor-1254 was purchased from AccuStandard, Inc. (New Haven, CT), dissolved in ethyl alcohol, and serially diluted in culture medium to obtain the desired concentrations. Three different concentrations of A-1254 (0.1,1.0 and 10 μg/mL) were studied, control culture dishes contained Minimal Essential Medium Eagle (MEM, Sigma Chemical Co., St. Louis, MO).

Culture Media

For embryo culture, MEM was supplemented with 0.75g/L penicillin-G sodium salt, 0.75g/L, streptomycin sulfate, 2.2g/L sodium bicarbonate, and 0.1091 g/L calcium lactate.

Brinster's medium for oocyte culture with 0.4% Bovine Serum Albumin (BMOC-3, Gibco, Grand Island, NY) was used for embryo collection. The same medium was used in the outer well of Falcon 3037 Organ Tissue Culture Dishes (Lincoln Park, NY). The MEM medium was prepared with cell culture grade distilled water (Gibco, Grand Island, NY) sterilized using a 0.22μm filter (Millipore, Bedford, NY) and aliquots were stored at 4°C in sterile 500ml plastic bottles. The pH of the medium was 7.30 to 7.45. Fresh media was prepared every third week.

Superovulation

Females were superovulated by an intraperitoneal (ip) injection of 10 IU of pregnant mare serum gonadotropin followed by 10 IU of human chorionic gonadotropin (hCG, both from Sigma Chemical Co., St. Louis, MO) 46-48 hours later.

Immediately following hCG injection, females were housed with males overnight at a ratio of one to one. Females were separated from males the morning after, checked for vaginal plugs, and placed back in individual boxes with food and water ad libitum.

Embryo Collection and Culture

Culture dishes were prepared using BMOC-3 (3ml) and MEM (1ml) in the outer and inner well respectively for each culture dish containing various concentrations of A-1254 and the control. The culture dishes when prepared were then equilibrated overnight in a humidified incubator with 5% CO_2 in air at 37°C.

Eight-cell embryos were collected from mated females at approximately 57 hours after hCG injections. Female mice were sacrificed by cervical dislocation, the abdominal cavity

exposed, and both oviducts and uterine horns removed and placed in a Costar cell culture cluster dish (Costar Corporation, Cambridge MA) containing 2 ml of BMOC-3 medium.

Embryos were removed by simply tearing the oviduct at several points along its length with a fine forceps, and flushing the isthmus of the oviducts through the uterine horn, using BMOC-3 media in a 1ml syringe attached to a 25 gauge (5/8 inch) hypodermic needle. Embryos were collected using a 10µl disposable micro pipet with a mouthpiece adaptor (Dade Diagnostic, Inc., Aguada PR). The embryos were then washed once in fresh MEM .

For every trial, three concentrations of Aroclor-1254 (0.1, 1.0 and 10.0 µg/mL) were prepared. Only embryos at the 8-cell stage not compacted and showing normal morphology and characteristics, with no signs of degeneration and/or abnormalities were used. From 6-10 embryos were used with every concentration in every trial for clear and easy identification of any changes in their development.

After the collection and embryo distribution, the culture dishes were placed in the incubator (5% CO_2 in air at 37°C) and observations were made every 24 hours up to 96 hours using a dissecting microscope to determine the number of embryos that developed further, indicated by the presence of compaction, morula, or blastocyst stages at the different concentrations of A-1254.

Statistical Analysis

Individual Chi square test (χ^2) using the Microsoft excel program, was performed on the data in order to determine if there was any significant difference ($P<0.05$) between the treatment and the control groups, due to the effect of Aroclor-1254.

RESULTS

Table 2 indicates the specific cell stage and time used for 8-cell embryo classification during the experiment.

Table 3 indicates the development to compacted 8-cell embryos at 24 hours of incubation. No statistical significant effects ($P > 0.05$) were found between the treatments; 0.1, 1.0, 10 μg/ml of Aroclor-1254, and control.

Table 4 indicates the development to morula stage at 48 hours of incubation. Statistically significant effects ($P < 0.05$) were found between the treatments; 0.1, 1.0, 10 μg/mL of Aroclor-1254, and control group. At this particular stage, there was a clear evidence of a dose response relationship.

Table 5 indicates the percent development to the blastocyst stage at 72 hours of incubation. A statistically significant effect ($P < 0.05$) was only found between the control and 1.0 μg/mL concentration of Aroclor-1254.

Table 6 shows the development of hatched embryos at 96 hours of incubation. No statistically significant effects were found ($P > 0.05$) between the three treatments; 0.1, 1.0 10. μg/mL of Aroclor-1254, and control group.

Table 7 indicates the degenerated embryos at 24 hours of incubation. No statistically significant effects ($P > 0.05$) were found between the three treatments; 0.1, 1.0 ,10. μg/mL of Aroclor-1254, and the control group.

Table 8 indicates the degenerated embryos at 48 hours, no statistically significant effects were found ($P>0.05$) in all the different treatments (0.1, 1.0 and 10. μg/mL) of Aroclor-1254).

Table 2: Specific cell stage and time used for 8-cell embryo classification during experiment.

Observation Period (Hours)	Days after Recovery	Stage of Development
24	1	Compacted 8-cell
48	2	Morula
72	3	Blastocyts
96	4	Hatched

Table 3: Embryo development to compacted 8-cell during the first 24 hours of in vitro culture.

Group	Total Embryos	Total Compacted	Percent Development
Control	69	44	64
0.1µg/mL/1254[1]	66	51	77
1.0µg/mL/1254[1]	62	42	68
10.0µg/mL/1254[1]	56	27	48
Totals	253	164	--

[1] Chi-square revealed no significant difference from the control ($P>0.05$).

Table 4: Embryo development to morula stage at 48 hours of in vitro culture.

Group	Total Embryos	Total Morulae	Percent Development
Control	44	40	91
0.1μg/mL 1254[1]	51	26	51
1.0μg/mL 1254[1]	42	18	43
10.0μg/mL 1254[1]	27	15	56
Totals	164	99	--

[1]Chi-square revealed significant difference from the control ($P < 0.05$).

Table 5: Embryo development to blastocyst stage at 72 hours of in vitro culture.

Group	Total Embryo	Total Blastocyts	Percent Development
Control	40	23	58
0.1μg/mL 1254	26	19	73
1.0μg/mL 1254[1]	18	18	100
10.0μg/mL 1254	15	10	67
Totals	99	70	--

[1]Chi-square test revealed significant difference from the control ($P<0.05$).

Table 6: Embryo development to the hatching stage at 96 hours of in vitro culture.

Group	Total Embryos	Total Hatched	Percent Development
Control	23	5	22
0.1μg/mL 1254[1]	19	4	21
1.0μg/mL 1254[1]	18	1	6
10.0μg/mL1254[1]	10	0	0
Totals	70	10	--

[1]Chi-square revealed no significant difference from the control (P>0.05).

Table 7: Embryos degenerated at 24 hours of in vitro culture.

Group	Total Embryos	Total Degenerated	Percent Degenerated
Control	69	6	9
0.1μg/mL 1254[1]	66	5	8
1.0μg/mL 1254[1]	62	5	8
10.0μg/mL 1254[1]	56	6	11
Totals	253	22	--

[1]Chi-square revealed no significant difference from the control (P>0.05).

Table 8: Embryos degenerated at 48 hours of in vitro culture.

Group	Total Embryos	Total Degenerated	Percent Degenerated
Control	69	7	10
0.1μg/mL1254[1]	66	8	12
1.0μg/mL1254[1]	62	6	10
10.0μg/mL1254[1]	56	9	16
Total	253	30	--

[1]Chi-square test revealed no significant difference from the control (P>0.05).

DISCUSSION

The results in this study demonstrated a significant effect of Aroclor-1254 on the development of the 8-cell mouse embryo, specifically at 48 hours for all the concentrations (0.1, 1.0, and 10 μg/mL) of 1254. Only the concentration of 1.0μg/mL of 1254 at 72 hours (blastocyst stage) indicated a significant effect. Aroclor -1254 also was detrimental for embryo development in a dose-dependent manner.

The criteria for the selection of the data to be analyzed statistically was based on time-specific stages; compacted 8-cells at 24 hours, morula at 48 hours, blastocysts at 72 hours and hatched at 96 hours. Establishing this selection criteria helped make a better judgment at the time of observations in order to determine if further development occurred based on morphological and structural changes in the 8-cell embryos.

The presence of other stages of development, in addition to the specific stage expected at that particular time, is due the fact that division of the blastomeres does not occur at the same phase for all the embryos (Bavister, 1987). Some of them could be developing at a faster or slower rate than other embryos that attained the appropriate cell stage at a specific time.

In the present investigation those, out-of phase embryos represented a small percentage of the total embryos. Another group of embryos observed were the degenerated, abnormal or dying embryos. These embryos showed a variety of morphological abnormalities such as fragmentation, irregular blastomeres, and cracked,

empty zonae pellucidae among many others. As expected, the number of degenerated embryos gradually increased, as the period of incubation increased from 24 to 96 hours.

There was no statistically significant effect, ($P>0.05$) on the 8-cell embryo during the first 24 hours of in vitro culture. At this stage the embryo is just starting the process of compaction. The blastomeres are distinct, therefore no gap-junction communications are established completely between the blastomeres.

These gap-junctions serve in the transformation of developmental information among blastomeres (Ziomek & Johnson, 1980). The lack of communication between the blastomeres of the 8-cell embryo could be a reason why Aroclor-1254 did not cause a significant effect at the three different treatments (0.1, 1.0, and 10 μg/mL) of Aroclor-1254.

Individual blastomeres could incorporate and metabolize a specific amount of the different concentrations of Aroclor-1254, at a specific rate independent of each other. The disadvantage of not having gap-junctional communications at this particular stage, may turn into an advantage for the embryo, granting some degree of protection (at 24 hours) against the toxic effect of Aroclor-1254.

At 48 hours, the results indicated a significant effect of the A-1254 affecting the morula stage at all concentrations. Previous to and after 48 hours there was no significant effect except for the 1.0μg/ml of 1254 at 72 hours (blastocyst stage), this could be an indication that the development of morula or the morula stage at 48 hours is more susceptible to the toxic effect of Aroclor-1254 . Additionally an indication of a dose response relationship was demonstrated with the number of 8-cell embryos that

developed into morula during the first 48 hours of invitro culture. As the concentration of Aroclor-1254 increase, the number of embryos developing into morula decreased.

Prior to the process of compaction the blastomeres are spherical and lack specialized intercellular junctions. The 8-cell embryos at 48 hours are fully compacted. During compaction cells flatten upon one another to maximize intercellular contact, establishing gap-junctional communications for the first time.

During the stage of compaction, polarization of the blastomeres first becomes evident (Ziomek & Johnson, 1980). This important process on the development of the 8- cell embryo occurs at the morula stage. In contrast to the embryos at 24 hours, the 8-cell embryos at the morula stage (48 hours) are more susceptible to the toxic effects from Aroclor-1254.

The main reason for this significant effect could be the establishment of the gap-junctional communications between blastomeres of the 8-cell embryo. As the individual blastomeres are exposed to the concentrations of Aroclor-1254, the chances of the other blastomeres receiving the same exposure of Aroclor-1254 are greater because all the blastomeres are connected through the junctions. Therefore, the toxic effect of Aroclor-1254 will end up affecting the entire embryo and its further development.

In the case of the blastocyst at 72 hours, only the concentration of 1.0μg/ml of Aroclor-1254 revealed a statistically significant difference ($P<0.05$). Considering that 100% development occurred only at this concentration (1.0 μg/mL of Aroclor-1254), and also that an effect is usually expected at higher concentration, suggests that this significant effect is basically coincidental.

Since there was a significant effect at 48 hours, most of the damage to the embryo that could affect its development due to toxic effects of Aroclor-1254, already occurred. Therefore, this could be an explanation, why no additional significant effect was indicated at the other two concentrations (0.1, and 10.0 μg/mL Aroclor-1254).

During the 96 hours observation period, there was no significant effect ($P>0.05$) on the percent of embryos hatched. During this particular stage of development, the embryo is ready to start the process of implantation. The results of non-significant effects were almost the results expected at this particular time, not because of the lack of toxicity of the Aroclor-1254, but mostly because, considerations such as variation in culture condition and media requirements for the implantation period were not applied in this experiment. The purpose of the observations at 96 hours, was to determine mostly if any significant change could be observed at the end of the pre-implantation period. As indicated in Table 6, the percent embryos hatched at 96 hours was much lower than any other period of observation.

Another mechanism by which Aroclor-1254 could affect the development of the embryos could be by reducing the number of gap junctions. Studies conducted by Krutovskikh et al., (1995), using four different tumor-promoting agents including Aroclor -1260, caused inhibition of intercellular communication. Krutovskikh et al., (1995), developed a dye-transfer technique to evaluate cell-coupling function in fresh liver slices, and used it to show that inhibition of intercellular communication is associated with rat liver tumor progression. Hemming et al., (1991) used the dye-transfer technique with rat liver white blood cells to measure the ability of polychlorinated biphenyl congeners to inhibit intercellular communication. Bager et al., (1994) demonstrated that PCB 1260

dramatically reduce gap junction protein expression in rat liver after 20 weeks of promotion treatment.

These studies were conducted using liver cells instead of embryos, however the function of the gap junction in both types of cells is believed to be involved in regulation of normal cell growth and differentiation. Further investigations need to be conducted in order to determine the specific mechanism by which Aroclor-1254 affects the development of the embryo.

There was no statistically significant difference ($P>0.05$) among the percentage of embryos degenerated from the 24-96 hours observation period, even when the percent of degenerated embryos gradually increased . Since the major effect on development of the 8-cell embryo occurred during the morula stage at 48 hours, the percentage of embryos degenerated before and after this particular period, was considered more important than the other observation periods. This information is indicated in Table 7 and Table 8.

The results in this study, contribute and support some of the most recent findings in other studies related to the toxicity of PCBs on the pre-implantation embryos. Kholkute et al. (1994b) examined the effect of A-1254 on the development of 2-cell embryos to the 4-cell stage at 48 hours, and they found that increasing the concentration of A-1254 in the culture medium significantly reduced the progression of 2-cell embryos to 4-cell stage or greater at 48 hours. The development of 4-cell embryos to expanded blastocyst was also significantly suppressed in 0.1, 1.0 and 10.0μg/mL of Aroclor-1254 groups.

Lindenau & Fischer, 1996 reported that Aroclor-1260, is embryotoxic in a dose-dependent manner . The study was conducted on one-day-old cleavage stages and three day-old rabbit morulae. This investigation further demonstrated the toxic effect of A-1254

at later stages other than 2-4 cell embryos. At these stages (2-4 cells) the mouse embryo is more susceptible to disruption on development by changes in the culture environment.

Some of the trials in this investigation were conducted for up to 120 hours and one up to 144 hours (data not shown), but no changes occurred. For this reason and also to have a more uniform set of data for the purposes of statistical analysis, data at 120 and 144 hours was not analyzed statistically. Also, these observations, were beyond the pre-implantation period.

SUMMARY AND CONCLUSIONS

PCBs have been demonstrated to be detrimental to reproductive functions in laboratory animals and wild mammalian species. Studies with Aroclor-1254 indicated adverse effects on early cleavage in the mouse embryo.

The 8-cell stage of the mouse embryo represents a very critical and important stage in the further development of the embryo. During the first 48 hours at the morula stage, the embryo undergoes major physical reorganization, including the process of compaction, the formation of gap-junctions and the process of polarization by which the components of the cell are re-organized.

This present in vitro study demonstrated the effect of Aroclor-1254 on the development of the 8-cell mouse embryo. The following conclusion were drawn from this investigation:

1. Aroclor-1254 did not affect the development of the 8-cell mouse embryo at 24 hours of culture in vitro, in all three concentrations tested..
2. Aroclor-1254 demonstrated a significant effect on development of the 8-cell embryo at 48 hours during the morula stage at the three concentrations tested (0.1, 1.0 and 10 μg/mL). In addition a dose response relationship was clearly observed.
3. Aroclor-1254 did not affect the development of the 8-cell mouse embryo at 72 hours during blastocyst stage at the concentrations of 0.1, and 10.0 μg/mL.

Only the 1.0 μg/mL concentration indicated a significant effect which may have been coincidental.

4. Aroclor-1254 did not affect the development of the 8-cell mouse embryo at 96 hours of culture in vitro, at all three concentrations tested.
5. Aroclor-1254 caused no significant effect, at all three concentrations tested, during the periods of observations (24-96 hours) in relation to the number of 8- cell embryos degenerated.

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APPENDIX A
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