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PCB EFFECTS ON MOUSE OOCYTES : THE POTENTIAL ROLE OF CUMULUS CELLS IN THE MEDIATION OF TOXICITY

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PCB EFFECTS ON MOUSE OOCYTES : THE POTENTIAL ROLE OF CUMULUS CELLS IN THE MEDIATION OF TOXICITY

By

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ABSTRACT

COMMERCIAL PCB EFFECTS ON MOUSE OOCYTES : THE POTENTIAL ROLE OF CUMULUS CELLS IN THE MEDIATION OF TOXICITY

By

Charles Ryan Greenfeld

Aroclor 1254, a commercial PCB mixture, has previously been shown to decrease mouse *in vitro* fertilization (IVF), by exerting toxicity upon the cumulus-enclosed oocyte through an unknown mechanism. This study examined the effects of A-1254 on zona pellucida (ZP) hardening in cumulus-free mouse oocytes. Oocytes were aged in the presence or absence of 1.0 or 10.0μg/ml A-1254, for 1, 3 or 6 hours. A-1254 neither induced nor inhibited ZP hardening. It did, however, reverse ZP hardening following 6 hours of aging. This indicates that A-1254 acted directly upon the ZP, altering its structural integrity, by means of a novel mechanism. IVF was also examined using cumulus-free mouse oocytes, aged for either 3 or 6 hours in the presence or absence of the same concentrations of A-1254. No reduction in fertilization was seen. These results suggest that cumulus cells may be involved in the mediation of PCB-induced toxicity.

DEDICATION

To my wife Lisa, for all your love and support, and for putting up with me through this sometimes arduous process. To my parents, thanks for the same, and for not giving up on me when it would have been easy to. And to Phish, whose music brought me solace often.

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Introduction

Polychlorinated biphenyls (PCBs) are a class of aromatic hydrocarbons that were used in many industrial processes from 1929 until their ban in 1977. Several inherent properties of these chemicals, including low reactivity, and resistance to degradation, made them attractive for industrial use. These same properties however, also make them highly persistent in the environment, and because they are lipophilic, they can be bioaccumulated in food chains. Because of this, PCBs will continue to be present in the environment, and in foods, and will continue to pose a health risk for many years to come (Battershill 1994).

Experimental evidence of the toxicity of PCBs in animals has been mounting since their detection in the environment in 1966. They have been found to cause problems ranging from hyperpigmentation of the skin to carcinogenesis (Norback and Weltman 1985; Guo et al. 1995). Due to extensive experimentally derived data, as well as correlative evidence of carcinogenicity of PCBs in animals, the United States Environmental Protection Agency (USEPA) has listed the chemicals as probable carcinogens (Kimbrough and Linder 1974; Bertazzi et al. 1987; Vater et al. 1995).

PCBs have also been shown to produce many reproductive problems. In mink, PCBs produce complete reproductive failure (Aulerich et al. 1973; Bleavins et al. 1980). They decrease pregnancies and litter size in the beagle, pig and rat (Earl et al. 1974; Brezner et al. 1974). They also decrease oocyte fertility *in vitro*. Huang and Chou (1994) found that in F1 female mice derived from PCB-exposed mothers, oocyte fertility was

reduced. Kholkute et al. (1994b) found that the commercial PCB mixture, Aroclor 1254, when present in the *in vitro* fertilization (IVF) culture media, reduced the percentage of mouse oocytes that were fertilized. The same response was produced following oocyte incubation in the presence of A-1254, while incubation of spermatozoa with A-1254 had no effect on fertility.

The mechanism responsible for decreased oocyte fertility following PCB exposure *in vitro,* has yet to be determined. "Hardening" of the zona pellucida (ZP) is one possibility. ZP hardening is a natural event in the fertilization process, following gamete fusion, and the subsequent cortical reaction (Kalab et al. 1991). It is responsible for the essential slow block to polyspermy. However, ZP hardening can occur prematurely during *in vitro* maturation in serum-free medium, or during *in vitro* aging, and can be induced by artificial activation of the oocyte (DeFilici and Siracusa 1982; Downs et al. 1986; Vincent et al. 1990). When it occurs prematurely, ZP hardening can cause infertility, as has been witnessed in human IVF programs (Ducibella et al. 1995). Assessment of ZP hardening can be accomplished *in vitro* by exposing the oocyte to proteolytic enzymes, or by performing IVF. It has been shown that following hardening, the ZP develops a resistance to dissolution by such enzymes, and also becomes impenetrable to sperm (DeFilici and Siracusa 1982; Gianfortoni and Gulyas 1985).

IVF, which is only one of many assisted reproductive techniques, has become well established as a research tool for screening reproductive toxicants. *In vitro* toxicity assays have many benefits over *in vivo* tests (Frazier 1992). These benefits include the speed at which the assay can be performed. With thousands of chemicals being introduced every

year, rapidity of toxicological assessment is very important and is a hallmark of *in vitro* tests. Cost is another benefit, with *in vitro* tests being considerably cheaper than their *in vivo* counterparts. Other benefits are that no pain is caused to live animals during *in vitro* tests, environmental conditions are controllable, variability between experiments is greatly reduced, and the interaction between organ systems is eliminated.

Toxicology is not the only field in which IVF has found application. It has been a staple in clinical human fertility programs ever since the birth of the first IVF human in 1978 (Fishel 1986). IVF is also receiving a great deal of attention from the field of conservation biology, where along with many other assisted reproductive techniques, it is being used in the conservation of endangered wild life. Some of these other techniques include, but are not limited to, *in vitro* maturation of both primordial follicles and immature oocytes, intracytoplasmic sperm injection, embryo transfer, and gamete/embryo cryopreservation and banking (Schroeder et al. 1988; Wildt 1989; Keefer 1990; Wildt 1991; Eppig and O'Brien 1996). It has also been recognized in the last few years that immature oocytes recovered from aged, sick, dying, or dead animals can be matured and fertilized in vitro, and can be used to obtain live offspring (Johnston et al. 1991; Schroeder et al. 1991; Eppig and O'Brien 1995). This latter technique has tremendous importance, as it can be used to salvage, or rescue genetic resources that would otherwise be wasted.

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 oocytes.

Literature Review

Mouse Development

The gestational period for the mouse fetus is in the range of 19-20 days (Hogan et al. 1986). During a natural ovulatory cycle, eight to twelve eggs are ovulated over a 2-3 hour period (Hogan et al. 1986). Ten hours after fertilization has occurred, the ovum has emitted its second polar body, and, by 24 hours, has reached the two-cell stage (Hogan et al. 1986; Theiler 1989). The morula stage is reached by two days post coitus, and development continues in the oviduct until day three when the embryo enters the uterus (Hogan et al. 1986; Theiler 1989). Implantation begins at 4.5 days. By 7.5 days, the amniotic cavity is sealed off, the neural plate is clearly delineated, both anteriorly and laterally, and the head process is developing (Theiler 1989). The heart is developing rapidly at 8 days, and the first seven somites are formed. The oral plate is formed at this point, and the brain is developing. Beginning in the region of the fourth and fifth somites, the neural folds close, and continue to do so both anteriorly and posteriorly. By nine days the heart is capable of maintaining minimal circulation, and placental circulation is being established. Both the forelimb and the hindlimb buds condense at 9.5 days. At the same time, the circulatory system further develops, as does the pancreas. Primordial germ cells can already be seen lined up at the genital ridge by ten days. The extremities and tail bud develop rapidly at 10.5 days, and the most cranial somites become more indistinct.

The genital ridge contains numerous gonocytes by 11.5 days. The lens vesicle has completed closure, and the cranial nerves and spinal ganglia are well developed. The lung buds have developed secondary and tertiary bronchi, and the stomach becomes greatly distended at 12 days. Sexual differentiation occurs at 13 days. Also at this time, the lungs are completely subdivided into lobes. The liver is well developed, and the kidney is developing. Hair follicles are present everywhere except the head by 14 days. By 15 days, the atrioventricular and semilunar valves are developed in the heart, and the arteries and veins have their final fetal configuration. The oral and nasal cavities are separated by palatal processes which fuse with the nasal septum. The ovary contains dividing gonocytes, and in the testes the seminiferous tubules are developed and differentiated. Vertebral ossification begins at 16 days, and blood cell production increases in the liver. At 17 days, many dividing oocytes have entered prophase I, and at 18 days the ovaries become attached to the lower poles of the kidneys, and are almost completely surrounded by the bursa ovarica.

The mouse is born at day 19-20. It is hairless, both its eyes and ears are closed. They remain that way for about two weeks. The vagina does not open until 6 weeks after birth, when the estrous cycle begins. Spermatogenesis is active at 24 days, which is also the time of weaning. The first successful mating can occur at 2-3 months. The mouse is full grown by 3-4 months (Theiler 1989).

PCBs : History, Production and Use

Polychlorinated biphenyls (PCBs) were first synthesized in 1881 by Schmidt and Schultz, but did not have industrial application until 1929 (Tanabe 1988). The Monsanto Corporation was the sole domestic producer of PCBs from this time until 1977 when the manufacture of PCBs was discontinued (Hutzinger et al. 1974; Brown et al. 1991). Their use had already been restricted once by this time, when Monsanto withdrew PCBs from the market in 1970, for all but closed systems use (Trout 1972). This voluntary withdrawal came after the identification of PCB compounds in environmental samples by Jensen and Widmark in 1966, and subsequent research showing widespread environmental contamination and possible health hazards (Norton 1972). Today in the United States, PCBs are no longer used commercially, but they are still present in many active electrical systems, and in many ecological systems throughout the world. PCB presence in the environment will continue for many years to come.

Production of industrially important PCB products was done by chlorination of biphenyl with anhydrous chlorine, using iron fillings or ferric chloride as a catalyst, until a desired endpoint of chlorine content was reached (Cook 1972; Hutzinger et al. 1974). This endpoint was determined by making specific gravity or softening point measurements on samples that were withdrawn during the chlorination process. Crude products were purified by treatment with alkali followed by distillation, to remove color and traces of both hydrogen chloride and ferric chloride. The end product would be a mixture of chlorobiphenyls containing different chlorine contents.

The Monsanto Corporation produced PCBs under the trade name of Aroclor® (Hutzinger et al. 1974). PCB mixtures were designated using a two number system. The first number represented the type of compound, for example PCBs were 12, and the second number represented the weight percentage of chlorine. Therefore, the name Aroclor 1254 signifies a PCB mixture with an average of 54% chlorine by weight. The commercially important Aroclor mixtures were A-1221, 1232, 1242, 1248, 1254, 1260, 1262 (Kalmaz and Kalmaz 1979), and Aroclor 1016, which does not follow the standardized nomenclature. A-1016 contains 42% chlorine by weight, and was introduced by Monsanto in 1971, as a biodegradable alternative to A-1242 (Bowers et al. 1975; Brinkman and DeKok 1980). In the United States, total production and use of PCBs from 1930-1975 exceeded 1.4 billion pounds (Brinkman and DeKok 1980).

Polychlorinated biphenyls contained many chemical properties, which will be discussed later, that made them useful for many industrial applications. These applications included : use as dielectric fluids in capacitors and transformers, industrial fluids for use in hydraulic systems, gas turbines and vacuum pumps, fire retardents, heat transfer systems, plasticizers, sealants, a component of the dye carrier in carbonless copy paper, and kill-life extenders in a variety of pesticides including Aldrin® and DDT (Trout 1972; Hutzinger et al. 1974; Gellert 1978). Prior to 1971, open-ended use, which included such uses as pesticide extenders, represented 26% of the total use in the United States, while nominally-closed, and closed system use represented 13% and 61% respectively (Brinkman and DeKok 1980). Many of these uses, especially open-ended, led to the introduction of PCBs into the environment.

PCBs : Chemical Properties

There are 209 theoretical PCBs. Any individual PCB is called a congener regardless of the degree or position of chlorination. A subclass of PCBs of a specified degree of chlorination (e.g. tetra- or pentachloro-) is a homolog, and a member of a homolog is an isomer (Norstrom 1988). There have been approximately 100 congeners identified in commercial PCB mixtures (Battershill 1994). Most of the individual congeners are solids at room temperature, whereas the commercial mixtures are either mobile oils (A-1221, A-1232, A-1242, A-1248), viscous liquids (A-1254), or sticky resins (A-1260, A-1262) (Cook 1972; Hutzinger et al. 1974). The solubility of PCB in water is very low and is inversely related to the degree of chlorination (Hutzinger et al. 1974). Volatility is also inversely related to the degree of chlorination, and is overall very low (Norton 1972; Kalmaz and Kalmaz 1979; Swain 1983). In general, PCBs have high thermal stability, and high resistance to both oxidation and hydrolysis in industrial settings (Hutzinger et al. 1974). These factors, combined with high general stability, inertness, and excellent dielectric properties led to their widespread industrial usefulness (Hutzinger et al. 1974). Ironically, these same factors contribute to their high environmental stability and persistence.

Of the 209 possible congeners, 20 can attain a coplanar configuration of the biphenyl ring system due to a lack of ortho- substitution (Tanabe 1988). Structure-function relationships show that only congeners substituted in both the para- and meta-

positions, and that lack ortho- substitution, can exhibit a maximum coplanar configuration and approximate the flat structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (Safe et al. 1985; Koopman-Esseboom et al. 1994). Congeners with high numbers of ortho-chlorines have reduced coplanarity and thus reduced toxicity (Koopman-Esseboom et al. 1994). The most active PCB congeners are : 3,3',4,4'-T_4CB; 3,4,4',5-T_4CB; 3,3',4,4',5-P_5CB; and 3,3'4,4',5,5'-H_6CB (Safe et al. 1985; Tanabe 1988). They all lack ortho- substitution, but are substituted at both para- positions and at 2 or more meta-positions, and are therefore approximate stereoisomers of both 2,3,4,7,8-P_5CDF, and 2,3,7,8-T_4CDD (Safe et al. 1985; Tanabe 1988). These coplanar congeners are present in low amounts in commercial mixtures and may be responsible for much of the overall toxicity of the mixture (Golub et al. 1991).

PCDFs have been identified as contaminants in Japanese PCB preparations, in all Aroclor preparations except A-1016, and have been found to be as high as 2 ppm in A-1248 (Bowes 1975; Goldstein et al. 1978; Wakimoto et al. 1988). Along with the PCDDs, the PCDFs are oxidation products and can be present in PCB preparations as contaminants. They can be created through industrial use (Barlow and Sullivan 1982). PCBs can be converted to PCDFs in temperatures as low as 300°C, and, in electrical or PCB fires, very complex mixtures of PCDFs along with a variety of other chlorinated aromatic compounds are produced (Erickson 1989). DeFelip et al. (1990) found both PCDFs and PCDDs to be present in residual fluid in a transformer following accidental leakage. Brown et al. (1988) also found PCDFs to be present in the dielectric fluids of

capacitors and transformers, but did not find evidence that they were produced during use.

PCBs : Environmental Contamination

PCBs were present in environmental samples as early as the 1950s. They were found in many specimens that were analyzed for the presence of chlorinated hydrocarbon pesticides, however they were ignored as unknown quantities until they were identified in 1966. Since then, PCBs have been found in the environment, and in organisms from pole to pole (Holmes et al. 1966; Koeman et al. 1969; Musial et al. 1974; Subramanian et al. 1983; Tanabe et al. 1983; Tanabe et al. 1987; Cummins 1988; Kipcic and Vukusic 1991; Bordet et al. 1993; Quemerais et al. 1994).

Cummins (1988) estimated that of the 1.2 million tons of PCBs in the world, 65% is either still in use in electrical systems, deposited in landfills or stored on land, and 31% percent has been released into the environment, the rest being degraded or incinerated. Environmental contamination by PCBs has occurred by : discharge in urban sewage effluent (Schmidt et al. 1971), leakage from electrical equipment, spills during their manufacturing and transport, vaporization or leaching from PCB containing formulations, improper handling and disposal (Nisbet and Sarofim 1972), stack emissions during incineration (Kocan et al. 1991), agricultural and municipal runoff, and by direct discharge into waterways by power plants (Kalmaz and Kalmaz 1979). Once in the environment, movement of PCBs predominantly occurs by atmospheric transport and

deposition which serves to distribute the chemicals worldwide (Bidleman et al. 1976; Kalmaz and Kalmaz 1979; Swain 1983; Tanabe 1988).

The most common route for PCBs to enter the food chain is by aquatic systems. After deposition into waterways following atmospheric fallout, PCBs tend to reside in the surface microlayer due to their hydrophobic nature (Kalmaz and Kalmaz 1979). Microorganisms and plankton are common food chain entry points, as they reside and feed in this region. This is where the greatest effects of PCB contamination are felt in the aquatic system, as they inhibit the growth and photosynthesis of phytoplankton at levels as low as 10-100 ppb (Norton 1972; Kalmaz and Kalmaz 1979). These organisms are essential to the functioning of aquatic systems as they provide oxygen and nutrients to the system.

Subsequent biomagnification occurs as consumers feed upon plankton, and the chemicals move up the food chain until they reach a top consumer. Top consumers generally have long lifespans, and can therefore be exposed to large doses of toxins over time. Among the most sensitive organisms to the toxic effects of PCBs are marine mammals, which can biomagnify the chemicals by factors as great as ten million times (Tanabe et al. 1984; Cummins 1988). These organisms are highly sensitive to PCBs because many have genetically influenced reductions in the ability to induce drug metabolizing enzymes (Cummins 1988). Animals with high-fat diets, such as marine mammals like polar bears, as well as humans, are at especially high risk. Polar bears rely on seal blubber as a major dietary component. Blubber can contain very high levels of PCBs and other organic toxins, as this is the tissue into which these chemicals tend to

partition. Levels of PCBs in the adipose tissue of polar bears increased four-fold from 1969 to 1984, and if this trend continues they will soon surpass the 50 ppm level designating them as toxic waste (Cummins 1988). High levels of toxins in marine mammals are also dangerous because they have fat rich milk, which facilitates their transfer to nursing young (Tanabe 1988).

PCBs : Accumulation, Metabolism, Elimination and Breakdown

As stated earlier, PCBs are persistent in the environment, highly lipophilic and can therefore be bioaccumulated (Bordet et al. 1993). This means that the toxin enters an organism, either by ingestion, or by diffusion across the body surface and becomes concentrated in tissues and organs that are high in lipids (Hutzinger et al. 1974). They do not necessarily remain in adipose tissue permanently. Rather, they can be mobilized into the plasma and travel to other parts of the body, eliciting toxicity, or they can be metabolized and excreted. Mobilization tends to occur during pregnancy, lactation and during periods of stress or starvation (Fuller and Hobson 1986).

Metabolism of PCBs is accomplished by hepatic drug metabolizing enzymes. Induction of these enzymes by PCB tends to follow two different patterns. One pattern is similar to that initiated by the drug phenobarbital (PB). PB induces the production of cytochrome P-450, the terminal oxidase of hepatic drug metabolizing enzymes, and enhances the metabolism of a wide variety of substrates (Goldstein et al. 1977; Goldstein et al. 1978). Congeners that follow this pattern are generally chlorinated in both the para-

and ortho- positions. The other pattern, of PCB metabolism, mimics that initiated by the carcinogenic polycyclic aromatic hydrocarbons. A prototype of this pattern is 3-methylcholanthrene, which induces both cytochrome P-448 and the associated aryl hydrocarbon hydroxylase (AHH) (Goldstein et al. 1977; Goldstein et al. 1978). Congeners of this type tend to be those that are chlorinated in both the para- and meta-positions. These congeners can assume a coplanar conformation, and can elicit a response similar to the highly toxic 2,3,4,7,8-pentachlorodibenzofuran, and 2,3,7,8-tetrachlorodibenzo-p-dioxin, but to a lesser extent (Safe et al. 1985).

Most PCB congeners follow the PB pattern of induction and are metabolized by cytochrome P-450 (Brown et al. 1989). The phenyl ring is hydroxylated at the 3- or 4-position by 3,4-epoxidation. This hydroxylation facilitates elimination of the molecule. Hydroxylation of the PCB congener can also follow an arene oxide intermediate (Barlow and Sullivan 1982). In general, the ease of metabolism is inversely related to the number of chlorines present (Hutzinger et al. 1974; Sugiura et al. 1976). It is also effected by the position of chlorine substitution. A high hydroxylation rate is attainable for congeners with no substitution at para- or meta- positions (Sugiura et al. 1976).

Biological half-lives and elimination of PCB are determined by the rate of metabolism. PCBs with low chlorine content have short half-lives and are eliminated fairly rapidly (Schmid et al. 1992). Niimi and Oliver (1983) found that following ingestion of one dose of PCB, containing 31 different di- to decachlorobiphenyls, by rainbow trout, half-lives were in the range of 5 days for the lower chlorinated congeners, whereas there was no apparent elimination of the more highly chlorinated congeners. In

human milk samples, levels of mono- and diortho congeners were significantly decreased over a four week postpartum interval, while the mean level of coplanar congeners containing para- and meta-, but no ortho-chlorines did not change (Koopman-Esseboom et al. 1994). Brown et al. (1991), in monitoring the levels and distributions of individual congeners in the sera of capacitor workers with chronic PCB exposure, found that most lower congeners were rapidly metabolized and eliminated. However, congeners with high numbers of chlorine persisted and could be used as near permanent indicators of exposure. In this same population, the observed half-lives ranged from 1.4 years for trichloro-, monoorthobiphenyls, to 12.4 years for hexachloro-, diorthobiphenyls (Brown et al. 1989).

Environmental dechlorination of PCB is slow at best. This is due in part to the fact that there are no known environmental chemical agents that have the negative reduction potentials needed to reductively dechlorinate the toxin (Brown et al. 1987). Bacteria can to some extent degrade PCB, but the ease of this process is inversely related to the degree of chlorination, as with higher organisms (Kalmaz and Kalmaz 1979). In recent years however, anaerobic microorganisms have been identified that can reductively dechlorinate highly chlorinated congeners, and aerobic bacteria have been identified that can oxidatively degrade more lightly chlorinated congeners (Abramowicz 1994).

Aroclor 1254

Aroclor 1254 is composed of a mixture of biphenyls containing an average of 4.96 chlorines per molecule (Hutzinger et al. 1974). It is a viscous liquid that contains 54% (w/w) chlorine and is composed of 11% tetra-, 49% penta-, 34% hexa-, and 6% heptachlorobiphenyls (Hutzinger et al. 1974; Linder et al. 1974). A-1254 was used in electrical capacitors and transformers, vacuum pumps, hydraulic fluids, adhesives, wax extenders, dedusting agents, pesticide extenders, inks, lubricants, cutting oils, and as a plasticizer in resins and in rubbers (Hutzinger et al. 1974). As with other commercial PCB mixtures (James et al. 1993), polychlorinated dibenzofurans have been detected as a contaminant in A-1254. Bowes (1975) detected the highly toxic contaminant in a preparation of A-1254 at a level of 1.7 ppm. A-1254 has been shown to produce toxic symptoms in non-human animal models (Earl et al. 1974; Merson and Kirkpatrick 1975; Brezner et al. 1984; Kholkute et al. 1994b; Arnold et al. 1995).

PCBs : Experimental Evidence of Toxicity

Since the discovery of PCBs in the environment, the toxicity of the chemicals in animal systems has been examined extensively. Virtually every animal studied is both susceptible to contamination and shows a toxic response following exposure. These responses are highly variable from species to species, and among tissues and organs within an individual animal. Responses of young and old individuals within a species are

also variable, with the young generally experiencing greater toxicity, as PCBs impair developmental processes.

This latter fact has serious implications, as young animals, both in utero and while nursing, can face high levels of exposure to PCB. Many studies have shown that PCBs are transferred from mother to young, either transplacentally to the fetus, or through milk to the nursing infant. Maternal transfer of PCBs to neonates occurs in many species, including, but not limited to, bats (Clark 1978), mice (Masuda et al. 1979), rhesus monkeys (Allen et al. 1980), rabbits (Seiler et al. 1994), and humans (Bailey et al. 1980; Jacobson et al. 1984). Effects related to this passage of PCBs from mother to young, include decreased egg hatchability in lake trout (Mac and Schwartz 1992), and in ring doves (Peakall et al. 1972), and they have been correlated with premature births in California sea lions (DeLong et al. 1973), and miscarriages in humans (Leoni et al. 1989). Earl et al. (1974) found that feeding beagle bitches 5 ppm/day A-1254, from the day of breeding, through parturition, caused decreased pregnancies and litter size at parturition and at two weeks postpartum. There was also increased resorptions and teratogenic abnormalities in these females. They also found the same response in pigs following oral administration of A-1254, beginning 21 days prior to breeding. In rats, the number of live pups per litter decreased following maternal exposure to A-1254 (Brezner et al. 1984). Arnold et al. (1995) found that 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 size for a given back length. The researchers also saw an increased incidence of nail lesions and gum recession in infants at 22 weeks

postpartum that had nursed with exposed females. In the human, infants born to women in the lake Michigan region who had chronic PCB exposure due to fish consumption, tended to be smaller in physical size, had reduced head circumferences and increased startle reflexes (Swain 1988). Infants born to women with high occupational exposure to A-1254, A-1242, and A-1016 have been shown to have decreased birth weights and gestational lengths (Taylor et al. 1984). However, the decreased birth weights were likely a result of the shortened gestation times. In another study, there was no relationship between serum PCB levels and preterm births (Berkowitz et al. 1996). The cause of the conflicting results in the above studies is not entirely clear, but may be a result of low maternal exposure in the latter study (Berkowitz et al. 1996).

PCBs elicit their toxicity by inducing numerous enzymes, including the hepatic and extrahepatic drug-metabolizing enzymes (Sanders et al. 1974; Safe et al. 1985). Their action in the liver causes increased weight of the organ in mice (Merson and Kirkpatrick 1975; Sanders and Kirkpatrick 1975), and rats (Linder et al. 1974). The induction of these enzymes, as well as the hepatic microsomal enzymes, has been shown to enhance the breakdown of steroid hormones. Sanders and Kirkpatrick (1975) found that testosterone levels in A-1254 exposed males was reduced, as was the number of sperm/mg testis. Jonsson et al. (1976) found a reduction in plasma progesterone in female rats following prolonged exposure to A-1242.

PCBs have been implicated to have mild estrogenic activity. Chronic exposure to A-1254 or Clophen® A60 leads to increased estrous cycle lengths (Orberg et al. 1972; Orberg and Kihlstrom 1973; Brezner et al. 1984). Gellert (1978) found that A-1221

significantly advanced the age of vaginal opening in rats and induced persistent vaginal estrus at a level of 10 ppm. In this study however, only a level of 1000 ppm A-1221 induced significant uterine growth, and the weight was only half that of a uterus from an animal given 1 ppb estradiol 17β. Other studies have found no estrogenic activity (Jonsson et al. 1976; Gellert and Wilson 1979).

Aside from hormone-like activities, PCBs have the capability to induce more acute effects in organ systems. These typically include thymic atrophy, immunotoxic responses, reproductive problems, porphyria and related liver damage (Safe et al. 1985). They can also produce skin problems including hyperpigmentation, and chloracne (Kuratsune et al. 1972). *In vitro,* PCBs induced aggregation of human platelets, and increased the secretion of serotonin from these cells (Raulf and Konig 1991). They have also been found to stimulate the production of inositol phosphates in polymorphonuclear neutrophils, and they are capable of activating protein kinase C and altering calcium homeostasis in rat cerebellar granule cells (Kodavanti et al. 1993; Kodavanti et al. 1994; Tithof et al. 1995). It was shown that ortho-substituted congeners are most potent in terms of activity in neuronal cells (Kodavanti et al. 1995). Aroclor 1242 produced complete reproductive failure in such sensitive species like the mink (Aulerich et al. 1973; Bleavins et al. 1980). Recently it was found that A-1242, A-1248 and A-1254 can all induce uterine muscle contraction, which could potentially play a role in premature births (Bae and Loch-Caruso 1996). Reproductive problems have also been shown to occur *in vitro*. Huang and Chou (1994) found that *in vitro* fertilization of mouse oocytes, taken from F1 females born to 3,3',4,4'-TCB exposed females, was reduced. Kholkute et

al. (1994b) found that at levels of 0.1μg/ml and higher, A-1254 had adverse effects on mouse IVF. These effects were produced only when the chemical was present in the IVF media, or following a six hour incubation of oocytes in PCB-containing media. No adverse effects occurred when spermatozoa were treated. This is cause for concern because PCBs, along with many other industrial and pesticide pollutants, have been found in human follicular fluid, in which the oocyte develops and matures. In a Canadian study of women undergoing *in vitro* fertilization, one group of women was found to have a mean PCB level of 4.08 ppb in follicular fluid (Jarrell et al. 1993). In two European studies, PCBs have been found at levels of up to 142 ppb in Austrian women, and up to 27 ppb in German women (Trapp et al. 1984; Baukloh et al. 1985). Seiler et al. (1994) found no reduction in fertilization rate *in vivo* in rabbits, following chronic oral exposure to A-1260, though they found that the chemicals accumulate to a high degree in both pre- and postimplantation embryos.

Human exposure to PCBs has been widespread, since the chemicals are both present and persistent in the food chain, and the fact that humans are top consumers, but there have been no definitive studies showing that adverse health effects are related to non-occupational exposures (Swanson et al. 1995). Nor has a consensus been reached about occupational exposures, but there have been adverse responses produced following exposure in these settings (Swanson et al. 1995). With many of these studies, there are confounding factors involved, such as multiple chemical exposures, especially in the occupational setting (James et al. 1993). Historically, there have been few instances of acute exposure involving large cohorts of people, unlike the large groups chronically

exposed by the situations discussed above. There have been two major poisoning episodes however : the Yusho epidemic in Japan, and the Yu-Cheng epidemic in Taiwan, both terms meaning oil disease.

The Yusho epidemic began in 1968, and affected 1,057 people (Kuratsune et al. 1972). The Yu-Cheng epidemic began in 1978 and affected over 2000 people (Lan et al. 1990; Guo et al. 1995). Both poisonings occurred due to the consumption of PCB-contaminated rice oil. There was also large consumption of PCDFs, which were created as a result of heating the oil. Common symptoms included increased eye discharge, swelling of the upper eyelids, chloracne, hyperpigmentation of the skin, and peripheral neuropathy (Kuratsune et al. 1972; Guo et al. 1995). A small sample of twelve babies born to Yusho women included 2 stillborn children, as well as 9 who had unusually gray/dark brown stained skin, five who had similar pigmentation of the gingiva and nails. Most had eye discharge (Kuratsune et al. 1972). Male school children affected with Yusho had decreased gains in both weight and height, but this was not seen in girls. Delayed development was also seen in children born 7-12 years after the Yu-Cheng epidemic, who were born to exposed mothers (Guo et al. 1994).

PCBs : Carcinogenic Potential

Many PCB mixtures, especially those with low chlorination (<50%), along with many individual congeners, have been seen to be minimally carcinogenic in many assay systems, while higher chlorinated mixtures (>50%) have been shown to be

hepatocarcinogens in rodents (Safe 1989). A-1254 produced adenofibrosis of the liver, as well as hepatocarcinoma in mice, following chronic oral exposure (Kimbrough and Linder 1974). Neoplastic lesions were produced in 170 of 184 livers, in rats chronically exposed to A-1260 (Kimbrough et al. 1975). Norback and Weltman (1985) found hepatomegaly, neoplastic nodules on the liver, and hepatocarcinoma to be produced in the rat following a long term feeding study of A-1260. The tumors produced did not metastasize to distant organs, nor did they invade blood vessels. They also did not increase mortality in the animals. A-1254 has also been shown to be a weak initiator in a two-stage system of mouse skin tumorogenesis (DiGiovanni et al. 1977), and a promoter of lung and liver tumors in the mouse (Anderson et al. 1994). Kerkvliet and Kimeldorf (1977) found that A-1254 can also function as an inhibitor of tumor growth. They found that A-1254, in a dose-dependent manner, inhibited the growth of tumors in rats inoculated with the Walker 256 carcinoma.

Data involving PCB-induced carcinogenesis in the human is inconclusive, and is for the most part correlative. Despite this, the Environmental Protection Agency (USEPA) has classified all PCB mixtures as probable carcinogens (Vater et al. 1995). Moore et al. (1994) claim that classifying all mixtures as having equivalent carcinogenic potentials has no scientific foundation and should be reconsidered. After evaluating seven studies in rats, involving PCB-induced liver tumorogenesis, they found that mixtures containing 60% chlorine consistently produced a significant increase of tumors, while mixtures containing 42 or 54% chlorine did not increase tumorogenesis.

As stated above, most evidence of PCB-induced carcinogenesis in humans is correlative, and has come primarily from studies of cohorts of electrical plant workers. Occupational cohorts, however, represent small populations with unusually high exposures (Ahlborg et al. 1995), and they often face exposure to many industrial solvents concomitantly to PCBs, which can add confounding factors to these retrospective studies (James et al. 1993). Nonetheless, many such studies have been undertaken in recent years. In a cohort of workers from an Italian capacitor manufacturing plant, where PCB mixtures containing 54 and 42% chlorine were used, the number of deaths due to cancer were higher than expected, with the lymphatic tissue and GI tract being the most common sites of tumorogenesis (Bertazzi et al. 1987). However, factors such as smoking history and socioeconomic indicators were not taken into account, so the role of PCBs in cancer initiation can neither be proved nor disproved. In a Canadian plant, where PCB-containing fluids were used in a small percentage of transformers that were produced, the incidence of pancreatic cancer was higher than expected (Yassi et al. 1994). In this cohort the exposure to mineral oil, which was refined from predominantly naphthenic base crudes, was much greater than their exposure to PCBs, so its role in carcinogenesis can not be discounted. There have also been other studies suggesting a correlation between PCB exposure and increased incidence of malignant melanoma, and cancer of the brain (Bahn et al. 1976; Sinks et al. 1992). Still more studies show no correlation between PCB exposure and increased cancer incidence (Gustavsson et al. 1986).

There have also been studies in humans undertaken to find a link between PCB exposure and breast cancer. In one study, adipose tissue samples were taken from women

with mammary carcinoma as well as women with benign disease (Falck et al. 1992). Mean levels of both PCB and p,p'-DDE were 50-60% higher in the tissues of women with breast cancer. Using logistic regression modeling, it was shown that PCB level was significantly correlated with breast cancer in all models, while p,p'-DDE was significant only when smoking was not included in the model. In another study, adipose tissue was sampled from deceased women with or without breast cancer, at autopsy, and also from living women with other breast disorders (Unger et al. 1984). The results showed no correlation between PCB levels and breast cancer.

Fertilization Events

Every day a species specific number of primordial follicles begin growth to the primary follicle stage. Follicle stimulating hormone (FSH), released from the pituitary gland, plays a supportive role in the continued growth and development of these follicles. Under continued FSH stimulation, folliculogenesis begins, enclosing the oocyte in layer upon layer of granulosa cells. At the level of the tertiary follicle the fluid filled antrum has formed, and follicular cells have completed differentiation into an inner layer of granulosa, and an outer layer of thecal cells, separated by the membrana granulosa. Through the process of development, less dominant oocytes in the cohort, which are not destined for ovulation, undergo atresia and are lost. The dominant follicle, or follicles in the case of multiple ovulators such as the mouse, continue to grow, and produce estrogen, until the luteinizing hormone (LH) surge occurs and stimulates ovulation. This surge also

stimulates the maturation of oocytes. Oocyte maturation is evidenced by germinal vesicle breakdown, and involves the progression of meiosis from prophase I to metaphase II, the extrusion of the first polar body, and accompanying cytoplasmic changes (Eppig 1993). Cytoplasmic maturation prepares the oocyte for activation, formation of pronuclei, and preimplantation development (Eppig and O'Brien 1995).

Prior to ovulation, during the growth of the immature oocyte, the zona pellucida (ZP) is produced (Dean 1992). The ZP is a thick extracellular coating which surrounds the plasma membrane in mammalian eggs (Wassarman and Mortillo 1991). It is responsible for the species-specificity of sperm binding, as well as the establishment of the slow block to polyspermy following gamete fusion. It also protects the preimplantation embryo as it travels through the oviduct (Wassarman and Mortillo 1991; Dean 1992). The ZP is composed primarily of three proteins, ZP1, ZP2 and ZP3, which represent 36%, 47%, and 17% respectively, of the total protein content (Bleil and Wassarman 1980; Wassarman and Mortillo 1991). At the peak of production, ZP protein synthesis represents 7-8% of the total protein produced in the oocyte, and this drops to zero by the time of ovulation (Dean 1992). The principle function of ZP1 is to maintain the structural integrity of the ZP, whereas ZP2, and ZP3 are involved in gamete interaction (Saling 1991). During oocyte maturation, the ZP also undergoes maturational changes, which include disaggregation in the texture of ZP proteins, which are necessary for sperm to penetrate the ZP (Tesarik et al. 1988; Rufas and Shalgi 1990; Henkel et al. 1995).

Fertilization begins when capacitated sperm contact the ZP. The ZP3 protein is the primary sperm receptor, and both binds the sperm and induces the acrosome reaction (Florman and Storey 1982; Bleil and Wassarman 1983; Shalgi et al. 1989; Wassarman and Mortillo 1991). For fertilization to be successful, the acrosome reaction must occur at the surface of the ZP (Saling 1991). Following induction of the acrosome reaction, ZP3 releases the spermatozoa, which then binds to ZP2, the secondary sperm receptor (Wassarman and Mortillo 1991). After binding to ZP2, the acrosome-reacted spermatozoa can penetrate through the ZP and enter the perivitelline space, where it then fuses with the plasma membrane. Here, the fusion of the gametes stimulates transient calcium increases, which trigger, among other things, the cortical reaction (Cuthbertson and Cobbold 1985). The cortical reaction entails the migration of the cortical granules (CG) to, and their fusion with, the plasma membrane of the oocyte (Wassarman and Mortillo 1991; Dean 1992). CG exudate, which includes proteinases and glycosidases, is released into the perivitelline space, and causes the alteration of ZP2 and ZP3, to $ZP2_f$ and $ZP3_f$ respectively (Barros and Yanagimachi 1971; Bleil et al. 1981; Kalab et al. 1991; Dean 1992). It is believed that this is due to protein cross linking via disulfide bonds (Zhang et al. 1991). After alteration, the spermatazoa receptors are inactive, and the essential block to polyspermy has been created, in a process termed the zona reaction (Schroeder et al. 1990). ZP "hardening", the result of the zona reaction, can also occur spontaneously during *in vitro* maturation in serum-free medium (Downs et al. 1986; Choi et al. 1987; Fujii et al. 1990; Kalab et al. 1991), as CG exocytosis occurs to a small extent during oocyte maturation (Ducibella et al. 1990), or due to oocyte aging (DeFelici and

Siracusa 1982; Dodson et al. 1989; Schiewe et al. 1995). ZP "hardening" manifests itself by creating both a loss of oocyte fertility (Gianfortoni and Gulyas 1985), and resistance to proteases (DeFelici and Siracusa 1982). It has recently been implicated as a cause of reduced success in human *in vitro* fertilization (IVF) programs (Ducibella et al. 1995).

Fusion of the gametes not only stimulates the cortical reaction. It also stimulates activation of the oocyte, which entails the resumption and completion of meiosis, as well as the extrusion of the second polar body (Kurasawa et al. 1989). At this point, the spermatozoa enters the cytoplasm of the now haploid egg, and releases its' genetic complement. The two haploid sets of chromosomes then join to form the first pronucleus, and mitosis, along with embryonic cell division, begins.

Materials and Methods

Animals

Female C57BL/6J, 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 $\pm$ 2 °C. Food and water were available ad lib. All females were at least 8 weeks of age and males were at least 12 weeks of age at the time of usage.

Aroclor 1254

A-1254 was purchased from Accustandard, Inc. (Lot #085-021, New Haven, CT) in neat form, dissolved in methyl alcohol. It was diluted in culture media to attain final concentrations of 1.0 and 10.0 μg/ml. These concentrations were chosen as they were previously reported to produce adverse effects in an *in vitro* fertilization system (Kholkute et al. 1994b).

Superovulation

Female mice were injected i.p. with 10 I.U. pregnant mares serum gonadotropin (PMS, Cat. #G-4877, Sigma Chemical Co., St. Louis, MO) between 4 and 5 PM. Forty eight hours later they were injected i.p. with 10 I.U. human chorionic gonadotropin (hCG,

Cat. #C-5297, Sigma Chemical Co.). Cumulus-enclosed oocytes were collected 17-19 hours following the hCG injection, as described below.

In Vitro Fertilization (IVF)

Brinster's medium for oocyte culture with 0.5% BSA, (BMOC-3, Cat. #11126-034, Gibco BRL, Grand Island, NY) was used for all sperm and oocyte collection and for IVF (Kholkute et al. 1994b). One milliliter of BMOC-3 was used in the inner wells of Falcon organ tissue culture dishes (Cat. #3037, Becton-Dickinson and Co., Lincoln Park, NY), and 3 ml were used in the outer wells. Media was prepared on the night before each trial and was equilibrated overnight in a humidified incubator under an atmosphere of 5% CO_2 in air at 37°C.

Females were superovulated as above. On the morning of the trial, one male was sacrificed by cervical dislocation. Two more males were sacrificed over the course of the experiment, 1-1.5 hours prior to use. The cauda epididymides were excised and placed into BMOC-3, where they were repeatedly 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 to occur. Approximately 30 minutes following sperm collection, 8-10 superovulated females were sacrificed by cervical dislocation. Their ovaries, oviducts and uteri were removed and placed into BMOC-3. Under a dissecting microscope, the cumulus masses were collected by teasing them from the swollen oviducts, and they were dispersed in 2mg/ml hyaluronidase (Cat. #H-2376, Sigma Chemical Co.). The cumulus-

free oocytes were rinsed and then 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 fifty to seventy five microliters of sperm suspension (~2-3 million sperm). There was a set of three groups that 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.

Twenty four hours after insemination, the oocytes were analyzed for fertilization. This was done visually under a dissecting microscope, and an oocyte was presumed to be fertilized if either two polar bodies were present, or if it had developed to the 2-cell stage (Kholkute et al. 1994b).

Zona Pellucida Hardening Assay

Following fertilization, and the subsequent cortical reaction, enzymes released into the perivitelline space alter the zona pellucida (ZP) proteins, ZP3 and ZP2, during the zona reaction. These proteins are responsible for primary sperm binding and the induction of the acrosome reaction, and secondary sperm binding respectively, and they are converted to $ZP3_f$ and $ZP2_f$ during this reaction (Schroeder et al. 1990; Wassarman and Mortillo 1991). This phenomenon inactivates these proteins, creating 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 fertilizability of unfertilized oocytes (Schroeder et al. 1990), and creates resistance to dissolution of the ZP by proteolytic enzymes such as α-chymotrypsin (DeFelici and Siracusa 1982). The present set of experiments were designed to determine whether a three or six hour exposure to A-1254 could induce ZP hardening, revealed by resistance of the ZP to proteolytic enzyme-induced dissolution, and if that was responsible for the reduced fertility of the oocytes *in vitro*.

The following protocol was identical for both the 3 and 6 hour exposure trials. Eight to ten female mice per trial were superovulated as above. On the morning following the hCG injection, females were sacrificed by cervical dislocation, and their reproductive tracts were removed. Cumulus masses were collected directly into Dulbecco's phosphate buffered saline (PBS, Cat. #14280-036, Gibco) at pH 7.8, which, along with all media used in these experiments, had been equilibrated overnight in a humidified incubator at 37°C under an atmosphere of 5% CO_2 in air. Following collection, cumulus masses were dispersed in 2mg/ml hyaluronidase in PBS. The cumulus-free oocytes were rinsed in PBS and transferred to one of the three 3 or 6 hour groups (the 1.0μg/ml A-1254 group, the 10.0μg/ml A-1254 group, or the control group) all of which contained 3mg/ml bovine serum albumin (BSA, Cat. #A- 2153, Sigma Chemical Co.) in PBS, or directly to a 200μl droplet of 2mg/ml α-chymotrypsin (Cat. #C-7762, Sigma Chemical Co.) in PBS. This final group, which was unaged, was the experimental control and was used to determine if effects seen were due solely to aging.

Following the 3 or 6 hour incubation period, oocytes were rinsed twice in PBS and then transferred to 200µl droplets (5-15 oocytes/droplet) of 2mg/ml α-chymotrypsin in PBS.

Once transferred to the α-chymotrypsin droplets, oocytes were assayed for the loss of their ZP. They were checked every five minutes under a dissecting microscope for the duration of the trial. ZP were considered solubilized when they had completely disappeared and the oocyte adhered to the bottom of the dish (Downs et al. 1986). Dishes were kept on a stage warmer at 37°C between examinations for ZP solubilization. The time required for 50% of the ZP to become solubilized, the $Lysis_{50}$, was used for comparison among groups (Gianfortoni and Gulyas 1985; Downs et al. 1986; Shroeder et al. 1990; Zhang et al. 1991).

Statistical Methods

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 (Freund and Wilson 1993).

Results

It was previously shown that following a 6 hour incubation of cumulus-enclosed oocytes, in the presence of 0.1, 1.0 or 10.0μg/ml A-1254, IVF was significantly reduced (Kholkute et al. 1994b). It was also shown that lowered fertilization rates were due solely to effects incurred by the oocyte, but that the viability of the oocytes was not compromised. The present study was undertaken to examine the functional effects of PCBs on mouse oocytes.

I chose to look at ZP hardening in cumulus-free oocytes cultured in serum-free medium, and hypothesized that PCBs induce the cortical reaction and subsequent ZP hardening. If this was true then the decreased fertilization rates seen previously could have been due to a failure of sperm to penetrate the ZP. Studies from other cell systems would seem to support this hypothesis : PCBs induce the formation of inositol phosphates in polymorphonuclear neutrophils, and both elevate intracellular calcium levels and activate protein kinase C in rat cerebellar granule cells (Kodavanti et al. 1993; Kodavanti et al. 1994; Tithof et al. 1995). All three of these processes occur naturally in the oocyte following gamete fusion, and are involved in the calcium-dependent signal transduction pathway that leads to oocyte activation and CG exocytosis (Kurasawa et al. 1989; Bement 1992). If PCBs act similarly in oocytes, as they do in these 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. Oocytes were aged for either 3 or 6 hours, following collection, in the presence of various concentrations (0.0, 1.0 or 10.0μg/ml) of A-1254.

These dose levels were chosen because they were shown to produce maximal reduction of IVF (Kholkute et al. 1994b). The values given are the mean $Lysis_{50}$s for eight trials. This value represents the time required to dissolve 50% of the ZPs in a group by α-chymotrypsin. Larger values indicate greater resistance to dissolution, which is an artifact of ZP hardening. Following three hours of incubation, A-1254 had no effect on ZP hardening. Although slightly lower, hardening in the PCB groups did not differ from the aged control ($p>0.05$). Both the 10.0μg/ml A-1254, and the aged control groups were significantly different from the unaged control ($p<0.05$), while the 1.0μg/ml A-1254 group approached significance. Increased resistance to ZP dissolution was gained in the aged control group due to limited CG exocytosis, which occurs spontaneously during *in vitro* culture in denuded oocytes (De Felici and Siracusa 1982). These results suggest that A-1254 has no effect on the cortical reaction.

The results from the 6 hour groups are also shown in Table 1. The $Lysis_{50}$ for the aged control was the same as for the 3 hour group, indicating that spontaneous CG exocytosis was completed by three hours of incubation. The $Lysis_{50}$s 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 result is unexpected because the cortical reaction had already triggered ZP hardening by three hours of exposure to A-1254, as evidenced by the increase in the $Lysis_{50}$ for the 3 hour groups (Table 1), in a nonreversible reaction.

The kinetics of ZP dissolution by α-chymotrypsin are shown in Figures 1 and 2. Figure 1 shows the curves for the 3 hour groups and illustrates that dissolution occurred

linearly over time. The rate of dissolution was fastest for the unaged control group. For the aged control, the rate of dissolution was rapid in the beginning, and slowed over time. Figure 2 shows that following 6 hours of incubation, dissolution also occurred linearly. It also shows that the rate of ZP dissolution was increased in the A-1254 groups, over both the unaged control, and the 3 hour A-1254 groups. It also shows that the rate of ZP dissolution for the aged control slowed greatly following 6 hours of aging.

To further clarify what was occurring, I chose to do a 1 hour aging trial. This would allow discrimination between the situation where A-1254 greatly increased ZP hardening in the short term, or had no effect at all on this phenomenon. The results of this trial are shown if Figure 3. The data in the graph is the ZP hardening relative to the unaged control, and was determined using the following equation, which was adapted from Gianfortoni and Gulyas (1985) :

$$\text{ZP Hardening (min)} = \text{Lysis}_{50} \text{ at time x} - \text{Lysis}_{50} \text{ at time 0.}$$

A-1254 had no effect on ZP hardening following 1 hour of exposure time. The Lysis_{50} for the 1.0μg/ml A-1254 group did not differ from the unaged control, and that for the 10.0μg/ml A-1254 was only slightly elevated. For the 1 hour aged control however, ZP hardening was evident. This shows 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 to occur until some time after one hour of culture.

The results for the IVF trials are shown in Table 2. IVF trials were carried out to evaluate the relationship between ZP hardening and oocyte fertilizability. 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. I felt it necessary to use cumulus-free oocytes, in order to determine what effect cumulus cells had on PCB toxicity in this system. Cumulus-free oocytes were aged for 3 or 6 hours, either in the presence or absence of 1.0 or 10.0μg/ml A-1254, as above, and were then inseminated. In contrast to Kholkute et al. (1994b), there were no differences in fertilization rates between groups ($p>0.8$). This indicates that PCBs may elicit their toxicity through a process involving the cumulus cells. Table 2 also shows 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 ($p<0.05$). This also differs from Kholkute et al. (1994a) who found that the percentage of degenerate oocytes increased with increasing A-1254.

Discussion

ZP hardening trials. In this study I examined the effects of a commercial PCB mixture, Aroclor 1254, on ZP hardening in cumulus-free mouse oocytes. The results suggest that A-1254 does not directly influence CG exocytosis-induced ZP hardening. At 1 hour, the $Lysis_{50}$ for the PCB groups was not elevated, while for the aged control it was. If A-1254 had a stimulatory effect on CG exocytosis than one would expect an increase in ZP hardening at one hour. These results do suggest, however, that A-1254 was inhibitory on spontaneous CG exocytosis at 1 hour, compared to the aged control where it began immediately. Spontaneous CG exocytosis did occur in the PCB groups by three hours, as shown by the increase in ZP hardening. This increase occurred to a lesser extent than in the aged control. If A-1254 had a complete inhibitory effect on CG exocytosis, one would not expect this increase at three hours.

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. CG exocytosis and the subsequent zona reaction are not reversible, so rather than influencing either of these reactions, the results suggest that A-1254 was acting directly upon the ZP to drop the $Lysis_{50}$s in these groups. Zhang et al. (1991) found that the addition of β-mercaptoethanol, a reducing agent, to serum-free medium during *in vitro* oocyte maturation, prevented ZP hardening. They suggested 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 (Wassarman and

Mortillo 1991), results in conformational changes of the ZP such that ZP1 becomes shielded from the extracellular environment. ZP1 acts as a filament crosslinker, maintaining the integrity of the ZP by interconnecting ZP2 and ZP3 (Greve and Wassarman 1985). It is also preferentially cleaved by α-chymotrypsin, resulting in the loss of interconnections between filaments and dissolution of the ZP (Greve and Wassarman 1985). It would appear that over time in culture, A-1254, by some unknown mechanism, alters the conformation of the modified ZP, making ZP1 more available to α-chymotrypsin, and thus decreases the resistance of the ZP to dissolution. If ZP hardening does in fact result from protein cross-linking by disulfide bonds, then A-1254 could act by disrupting these bonds. Bond disruption and the alteration of membrane structural integrity represents a novel mechanism of action of PCBs.

The fact that A-1254 does not stimulate CG exocytosis is surprising, based on the activity of PCBs and similarly acting chemicals in other cellular systems. 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). Compounds such as 4β-phorbol 12,13-didecanoate (4β-PDD), and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), which are biologically active phorbol esters; and sn-1,2-dioctanoyl glycerol (diC_8), a diacylglycerol, are PKC activators in the mouse oocyte and induce both CG exocytosis and ZP modifications (Endo et al. 1987; Ducibella et al. 1993).

Others have reported activities of PCBs that would further suggest their role in CG exocytosis in the oocyte. Tithof et al. (1995) found that in polymorphonuclear neutrophils, A-1242 stimulated the production of inositol phosphates, among them inositol 1,4,5-trisphosphate (IP_3). IP_3 both stimulates calcium release from intracellular stores, and activates PKC. It has been shown to initiate CG exocytosis and oocyte activation (Cran et al. 1988; Ducibella et al. 1993). Intermediate weight Aroclor mixtures (1248, 1254, and 1260) were shown to enhance high affinity binding of ryanodine to ryanodine-sensitive calcium release channels, in the membrane of the sarcoplasmic reticulum in both skeletal, and cardiac muscle cells (Wong and Pessah 1996). The PCB mixtures were also shown to decrease the potency of inhibition of calcium release, by calcium, through negative feedback. Ryanodine receptors have been located in both mouse and bovine oocytes, and microinjection of ryanodine into mouse oocytes stimulates CG exocytosis and ZP modification, though to a lesser extent than IP_3 (Ayabe et al. 1995; White and Yue 1996). The fact that PCBs can induce IP_3 production, and can

enhance ryanodine binding to calcium-sensitive release channels, further argues for their role in stimulating CG exocytosis.

There are several potential reasons to explain why PCBs do not induce CG exocytosis, in spite of the evidence presented above which suggests otherwise. The first is simply that PCBs may act differently among different cell types. Differences in the way that cells respond to toxic insult can lead to alterations in the way that PCBs behave. In rat cerebellar granule cells, only ortho-substituted congeners, which are unable to assume a coplanar configuration, are capable of inducing PKC activity (Kodavanti et al. 1995). The same is true in polymorphonuclear neutrophils, with regards to inositol phosphate production (Tithof et al. 1995), and in skeletal and cardiac muscle, with regards to stimulating high affinity ryanodine binding (Wong and Pessah 1996). However, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a coplanar compound and structural analogue to PCB, was found to stimulate PKC activity in plasma membrane preparations from rat livers (Bombick et al. 1985).

Species-specific differences with regards to PCB behavior, have also been demonstrated. Identical congeners were found to produce different functional responses of PKC from rat and mouse brain extracts (Shukla and Albro 1987). In mice, PCBs caused increased binding of phorbol esters, an indicator of PKC activity, but decreased histone phosphorylation. In rats, PCBs caused decreased phorbol ester binding, but increased histone phosphorylation. Species-specific differences in the behavior of other PKC activators have been demonstrated. In murine oocytes, TPA was able to induce transient calcium oscillations, which occur following gamete fusion, and sustain them for

up to three hours (Cuthbertson and Cobbold 1985). However, in the hamster oocyte, calcium oscillations induced by guanosine-5′-*O*-(3-thiotriphosphate) (GTP[S]), were inhibited by TPA (Swann et al. 1989). This led the researchers to suggest that there may be species-specific differences in the mechanisms of control of the calcium-dependent signal transduction pathway. All of this discussion serves to illustrate that behaviors seen in one cell type are not necessarily produced in another cell type. The mouse oocyte could be an example of this with regards to the induction of CG exocytosis.

Another reason why PCBs did not induce CG exocytosis may be a result of their effects on calcium homeostasis. As discussed above, PCBs have been shown to induce large and sustained increases in intracellular calcium levels (Kodavanti et al. 1993). 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 activation events, including meiotic resumption, and the extrusion of the second polar body (Kline and Kline 1992). It has been shown that TPA can induce similar calcium oscillations in mouse oocytes and can parthenogenetically activate them (Cuthbertson and Cobbold 1985). Ethanol, dimethylsulphoxide, and calcium ionophore (A23187) can induce CG exocytosis and ZP hardening (Gulyas and Yuan 1985; Ducibella et al. 1988; Vincent et al. 1990). While these agents do not induce calcium oscillations, they do induce a single transient rise in intracellular calcium levels (Cuthbertson et al. 1981; Eusebi and Siracusa 1985; Miyazaki 1991). If PCBs do act in the oocyte, as they do in rat cerebellar granule cells, and cause an increase in intracellular

calcium levels in a nontransient manner, then CG exocytosis would potentially be inhibited. This could occur due to a desensitization, or inhibition of the enzymes involved in the calcium dependent signal transduction pathway, or by uncoupling signals between members in the pathway. Whatever the mechanism may be, a nontransient rise in intracellular calcium could lead to a failure in the initiation of activation.

The lack of cumulus cells in this study could also explain why A-1254 did not induce CG exocytosis. PCB-induced toxicity could potentially be mediated by a mechanism requiring the oocyte to be cumulus-enclosed. This possibility will be discussed below.

IVF trials. The IVF results that I obtained were different from those previously reported (Kholkute et al. 1994b). In that study it was shown that A-1254 significantly decreased IVF in cumulus-enclosed oocytes. A-1254 had an inhibitory effect when present in the IVF media, and also following a six hour incubation period of cumulus masses in its presence. It was also shown that A-1254 increased the percentage of degenerate oocytes (Kholkute et al. 1994a). Neither of these effects were replicated in this study using cumulus-free oocytes. Compared to the control group, fertilization was not affected at either 1.0 or 10.0μg/ml A-1254 dose level, nor was it affected by duration of incubation prior to insemination. While the percentage of fertilized oocytes in this study was lower than that reported by Kholkute et al. (1994b) (49.6% vs. 79.8% respectively), it was not different from values reported elsewhere for cumulus-free oocytes (Saeki et al. 1994). The percentage of degenerate oocytes was also unaffected by

A-1254 in this study. In fact, the percentage of degenerate oocytes in the unaged control group was the highest, and was significantly higher than both of the 6 hour A-1254 groups. The reason for this increase in degenerate oocytes in the unaged control is unknown.

The fact that A-1254 did not affect IVF in cumulus-free oocytes gives further support for the notion that it did not induce CG exocytosis and subsequent ZP hardening. If it had, than fertilization should have been decreased in the treatment groups, due to a failure of sperm to penetrate the ZP. The results also suggest that A-1254 did not induce a rise in intracellular calcium. If it had induced this rise, than the gamete fusion-induced calcium oscillations would not have been able to trigger activation events, and fertilization would not have occurred. The same fertilization percentages could have been obtained if the oocyte had become desensitized to PCBs with regards to inhibition of the calcium buffer systems, and became able to lower intracellular calcium levels. Fusion-induced calcium oscillations would have then been able to trigger activation. The same percentages could have also been obtained if it is assumed that what is important in triggering activation is the attainment of some threshold calcium level, rather than a relative change in intracellular calcium concentration. If the PCB-induced calcium rise was below the threshold value, than gamete fusion could potentially elevate calcium beyond the threshold level, and activation would occur.

The potential role of cumulus cells in PCB toxicity. Based on the results from both IVF and the ZP hardening assay I suggest a role for the cumulus cells in PCB-

induced toxicity in the oocyte. This suggestion is made due to the fact that A-1254 neither stimulated nor inhibited CG exocytosis, nor did it lower IVF in cumulus-free oocytes. I did not examine the effects of A-1254 on ZP hardening in cumulus-enclosed oocytes, though it seems warranted, and should be examined in the future. The effects of A-1254 on IVF in cumulus-enclosed oocytes have been examined, and the results demonstrated adverse effects on fertilization.

There are several mechanisms by which cumulus cells could be involved in the mediation of PCB-induced toxicity. The first is that they could aid in PCB accumulation within the oocyte. PCBs would enter the cumulus cells with ease, then pass into the oocyte through gap junctions. This is unlikely, as the ZP is a highly porous membrane, allowing passage of even small viruses (Gwatkin 1967), and due its lipophilicity, PCB should find ready access into the oocyte. Another possibility is that the effects of PCBs are elicited by a second messenger, the production of which is stimulated in the cumulus cell, which is passed to the oocyte through gap junctions. This messenger could then produce toxicity. Conversely, PCB could inhibit the production and transfer of a factor required by the oocyte, and the lack of this factor would be the cause of toxicity.

The regulation of PKC represents another potential site for cumulus cell mediated effects. Colonna et al. (1989) found that a 74-kDa phosphorylated protein, the function of which is unknown, was present only in granulosa cell-enclosed oocytes. They proposed 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 therefore CG exocytosis, and could potentially cause an

excess of aneuploidy, as the slow block to polyspermy would not be created. This could explain the results in Kholkute et al. (1994a), in which it was found that A-1254 caused an increase in the percentage of degenerate oocytes. The potential for PCBs to inhibit intercellular communication by gap junctions has been demonstrated in rat liver cells (Hemming et al. 1991; Krutovskikh et al. 1995). The loss of intercellular communication between the oocyte and granulosa cells, would then be responsible for the adverse effects on IVF. While the oocyte does not require signaling from cumulus cells, it does play a large role in oocyte functionality, as shown by a reduction in fertilization by 30% in comparison to Kholkute et al. (1994b). Similar reductions in fertilization, between cumulus-enclosed and cumulus-free oocytes, have also been reported elsewhere (Saeki et al. 1994). PKC activity in the oocyte is still functional without cumulus-signaling, since fertilization can occur, but the lack of signaling may cause the oocyte to be less responsive to fertilization stimuli, as well as to toxins such as PCB.

The loss of communication between the cumulus cells and the oocyte, could also be deleterious in and of itself. It is unknown whether it is more harmful to the oocyte to inhibit communication between itself and attached cumulus cells, than it is to remove them. That is, in both cases cumulus signaling is absent, but it is unknown if the maintenance of contact with the cumulus cell creates more harm. If it is, than PCBs could be toxic to the oocyte simply by inhibiting communication.

There is one final potential mechanism that needs to be addressed as it helps to explain both the role of cumulus cells in mediating PCB toxicity, as well as some of the results obtained in Kholkute et al. (1994b). In that study, it was shown that IVF was

decreased to a greater extent (20-25%) when A-1254 was present in the IVF medium, than when cumulus-enclosed oocytes were incubated for 6 hours in its presence, and then inseminated in PCB-free media. A possible explaination is that upon initial exposure of cumulus-enclosed oocytes to A-1254, calcium levels are increased in the oocyte, due to signals induced in the cumulus cells by A-1254. Inhibition of the oocytes' buffering system would also occur, and so the rise in calcium would be nontransient. Thus far, the mechanism is in agreement with the activity of PCBs in other cell types (Kodovanti et al. 1993). When inseminated in the presence of A-1254, gamete fusion-induced calcium oscillations would not be able to induce activation events, due to elevated intracellular calcium levels. This would yield low fertilization rates, and a high percentage of aneuploidy in these oocytes. With regard to the 6 hour incubation of cumulus masses, the initial rise in calcium also occurs. However, over time through the inhibition of intercellular communication, the inhibitory effect of A-1254 on the oocytes' buffer system would slowly cease. This would allow the intracellular calcium levels to slowly return to normal, and this could be what is responsible for the 20-25% improvement in fertilization rates following a 6 hour exposure prior to insemination.

While all of these mechanisms are speculative, they deserve attention. Examining the effects of PCBs on the mammalian oocyte is important, as PCB exposure continues to pose a threat to the health of all animals, and has the potential to create severe reproductive and developmental problems.

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APPENDICES

APPENDIX A

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).

Group	$Lysis_{50}$ (min) Mean ± SEM		
	0 Hour	3 Hour	6 Hour
Control	27.19 ± 4.40 (322)	45.00 ± 7.44 (154)	45.00 ± 4.53 (130)
1.0µg/ml A-1254		40.62 ± 4.95 (142)	24.38 ± 5.30 (148)
10.0µg/ml A-1254		42.50 ± 5.43 (135)	26.25 ± 4.60 (148)

The Kruskal-Wallis test showed an overall significant difference between groups ($p<0.05$). 0hr control vs. 3 and 6hr controls, and 10.0µg/ml A-1254 ($p<0.05$). 6hr 1.0 and 10.0µg/ml A-1254 vs. 3 and 6hr controls ($p<0.05$).

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

Group	Total Number of Oocytes	Number of Fertilized Ooctes (%)	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 ($p<0.05$).

APPENDIX B

APPENDIX B

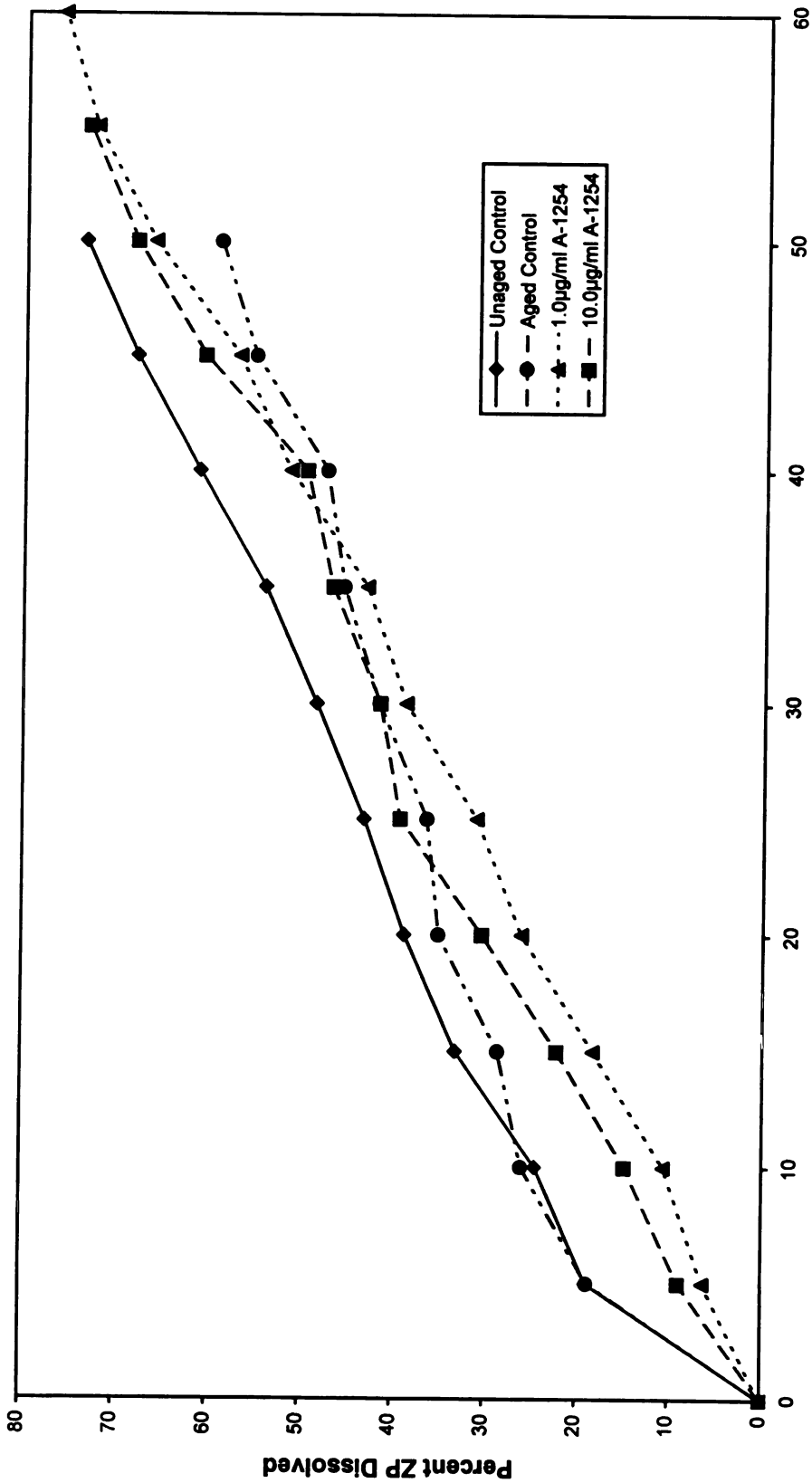

Figure 1. Rate of zona pellucida dissolution following a three hour incubation

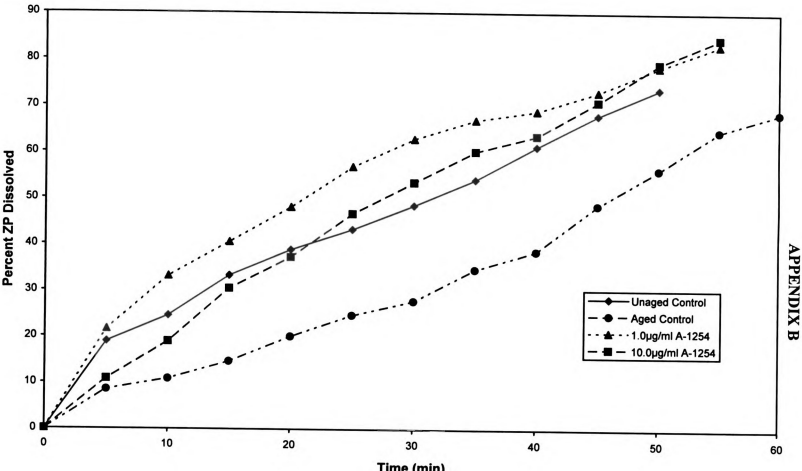

Figure 2. Rate of zona pellucida dissolution following a six hour incubation

APPENDIX B

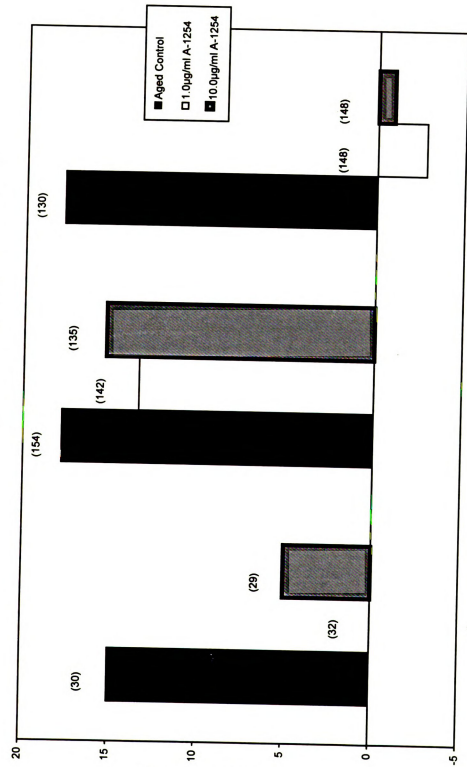

Figure 3. Zona pellucida hardening following various incubation times. (Values in parentheses are the number of oocytes).

APPENDIX C

APPENDIX C

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2. Dukelow WR, Greenfeld CR, Hernandez O, Clemens LG, Chung Yu-Wen, Kaneene JB. 1996. Toxic chemical influences on *in vivo* and *in vitro* reproduction. Proc Forum on Environmental Remediation and Environmental Toxicology. Lansing MI. (Abstract).

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