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PHYSIOLOGICAL AND TOXICOLOGICAL EFFECTS OF CAFFEINE, HOECHST 33258 AND TOLUENE ON MOUSE FERTILIZATION IN VITRO

By

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ABSTRACT

PHYSIOLOGICAL AND TOXICOLOGICAL EFFECTS OF CAFFEINE, HOECHST 33258 AND TOLUENE ON MOUSE FERTILIZATION IN VITRO

By

Frank D. Yelian

Studies were conducted to test the some physiological and toxicological effects of caffeine, DNA-specific fluorochrome Hoechst 33258 and toluene on B6D2F1 mouse fertilization in vitro. In order to understand the chemical effects on the different stages of fertilization, the direct influences of the chemicals on epididymal sperm motility, sperm capacitation and acrosomal reaction, single gamete cell intracellular Ca^{++} concentration, fertilization rate, and embryo development were observed.

The results demonstrated that caffeine (0.1 to 10 mM) significantly increased sperm motility. The stimulatory effect was Ca^{++}-dependent. Caffeine at the 0.1 and 1.0 mM levels had no effect on fertilization, but 10 mM of caffeine significantly decreased fertilization rate. The chlortetracycline (CTC) fluorescence assay was used to determine sperm acrosomal status, the result demonstrated that caffeine (10 mM) did not affect sperm capacitation or acrosomal reaction.

DNA-specific fluorochrome Hoechst 33258, as a non-toxic

stain, has been used to detect DNA distribution and synthesis in living cells, it is also used to detect early fertilization. The toxicological study demonstrated that 20 and 100 ug/ml decreased the fertilization rate. The results also demonstrated that Hoechst 33258 at 10 ug/ml had no effect on fertilization, but gametes incubated with 10 ug/ml or higher levels of the stain inhibited embryonic development.

Previous mouse in vivo studies indicated that toluene administrated by gavage increased the embryonic mortality. The present in vitro study also showed that a concentration of toluene higher than 8.67 ug/ml not only decreased sperm motility and inhibited fertilization, but also increased the percent of embryo degeneration.

To understand the mechanism of chemical effects on sperm motility, the ACAS 570 interactive laser cytometer was used to measure the single gamete cell and embryo calcium distribution and dynamic change. The results demonstrated that caffeine (10 mM) elevated sperm midpiece intracellular Ca^{++}, but had no effect on the sperm head. In the sperm treated with 8.67 ug/ml of toluene, no significant calcium change was observed.

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INTRODUCTION

We live in a chemical world. Our daily life involves many different chemical substances. Caffeine and toluene are the examples, and we must consider their effects on our health.

Early studies indicated that caffeine is a phosphodiesterase inhibitor, which increases intracellular cAMP levels and stimulates sperm motility. Caffeine has been used to treat low motility sperm from some infertile males or after cryopreservation. One objective of the present in vitro study is to use mouse epididymal sperm and oocytes to observe the direct effects of caffeine at different concentrations on sperm motility, intracellular calcium concentration, capacitation, acrosomal reaction, and fertilizing ability. I also wish to understand the mechanisms involved in sperm motility regulation and to determine the toxic level of caffeine on mouse sperm function in vitro.

DNA-specific fluorochrome Hoechst 33258, as a non-toxic stain, has been used in measuring cell density and proliferation; assessing DNA distribution and synthesis; observing sperm-egg fusion and preimplantation embryonic development. No data are available on the toxic level of this stain on gamete cells. Another objective of these studies was to observe the toxic effects of different concentrations of the stain, from 1 to 100 ug/ml, on mouse sperm motility, fertilizing ability and embryo development in vitro. From such

studies, we could establish the cellular toxicity of Hoechst 33258, which would benefit future studies on living cells.

Toluene is an aromatic hydrocarbon, commonly used in the industry,in spray paints and glues. According to the United States EPA reports, toluene has been detected in municipal water supplies at levels ranging from 0.1 to 11 ug/l. There are a number of case reports on pregnant women's exposure to toluene causing infant malformations and congenital defects. A previous in vivo study on the mice also demonstrated that toluene could induce embryonic lethality i.e have teratogenic effects on the fetus. The present study is designed to test the direct toxic effect of toluene on sperm motility, in vitro fertilization and embryonic development.

To understand the mechanism of caffeine and other chemicals effects on sperm motility and fertilization, the studies are also designed to test the calcium involvement. This will include comparing the caffeine effect on sperm motility in normal BMOC-3 medium and Ca^{++}-free BMOC-3 medium, measuring intracellular Ca^{++} concentration, distribution in a single sperm, oocyte and embryo, detecting the chemical effects on Ca^{++} dynamic change in the sperm cells and in oocytes during sperm penetration.

The ACAS interactive laser cytometer is a very sensitive instrument which allows measurement of single living cell intracellular Ca^{++}, pH, DNA synthesis, membrane transport, and

detect cell-cell communication. The ACAS 570 has been applied for many different cell studies, including lymphocytic, somatic, cancer, and granulosa cells.

PHYSIOLOGICAL AND TOXICOLOGICAL EFFECTS OF CAFFEINE, HOECHST 33258 AND TOLUENE ON MOUSE FERTILIZATION IN VITRO

LITERATURE REVIEW

CAFFEINE AND SPERM MOTILITY

Garber et al. (1971a; 1971b) reported that caffeine stimulated bovine epididymal and ejaculated spermatozoa motility. They found that caffeine-treated spermatozoa maintained the initial percentage of motility for at least 4 hr at 37°C whereas in the untreated sperm samples, 50% of sperm that were motile initially became immotile during 4 hr storage.

Since then, many researchers have studied the effects of caffeine and other methylxanthines on sperm motility and velocity in different species. Most of results demonstrated that caffeine does increase and even prolong sperm motility (Burge, 1973; Schoenfeld et al., 1975; Fraser, 1979; Moussa, 1983; Rees et al., 1990). However some studies have failed to confirm any stimulatory effect for caffeine (Dougherty et al., 1976; Markler et al., 1980). The effects on sperm velocity were controversial. One study (Markler et al., 1980) used combined multiple-exposure photography and supravital staining to demonstrate that caffeine had no influence on sperm

velocity. This was later confirmed by others (Levin et al., 1981; Aitken et al., 1983; Moussa, 1983). However Ruzich et al. (1987) used Cell-Soft computer analysis methodology and concluded that caffeine produced not only an increase in the percent motile sperm but also an increase in sperm velocity.

The loss of motility and the concomitant loss of fertilizing potential exhibited by human spermatozoa after cryopreservation is a major problem for artificial insemination and in vitro fertilization programs (Serres et al., 1980; Aitkien et al., 1983; Cai and Marik, 1989). The possible use of caffeine to stimulate the motility of cryopreserved human spermatozoa has been suggested by the positive results obtained by Barkay et al. (1977) and Schill et al. (1979). However, results in terms of pregnancy rates are disappointing; the most likely explanation for this appears to be the occurrence of an alteration in the ultrastructure of the sperm head, as observed with scanning electron microscopy of sperm incubated with caffeine (Harrison et al., 1980). A critical assessment of the response to caffeine of human sperm motility was done by Traub et al. (1982). They concluded that the widely documented effect of caffeine on human sperm motility is of a transitory nature and in keeping with ultrastructural damage.

The concentration of caffeine used is very important. Levin et al. (1981) used caffeine at levels from 0.012 to 2.0

mM and observed a dose-dependent increase in sperm motility. The ED50 for motility stimulation was 300 uM of caffeine. A significant increase in motility was observed with 60 uM, and maximal stimulation occurred with 1 mM. Moussa (1983) used six different levels of caffeine to assess the effect on human sperm motility. In levels of 3 and 6 mM, caffeine significantly increased the motility, but had no effect on sperm velocity. At a level of 120 mM, caffeine causes complete immobilization of human spermatozoa (Moussa, 1983).

The mechanism of action of caffeine on spermatozoa motility is still not clear. Caffeine inhibits cyclic nucleotide phosphodiesterase (Hardman et al., 1971), enzymes which are involved in spermatozoa glycolysis and consequently increases the intracellular cyclic 3':5'-adenosine monophosphate (cAMP) and cyclic 3':5'-guanosine monophosphate (cGMP) concentrations (Hicks et al., 1972). This is the probable site of caffeine action. Levin et al. (1981) quantitatively analyzed the effects of caffeine on sperm motility and cAMP phosphodiesterase. They found that the phosphodiesterase activity of sperm homogenates displayed linear kinetics with a Vmax of 0.5 nmoles/mg of protein per minute and a Km of 125 uM. Caffeine (0.1 to 40 mM) produced a dose-dependent inhibition of sperm phosphodiesterase, displaying a Ki of 1.2 mM. The results suggested that caffeine may stimulate sperm motility by a mechanism other than

phosphodiesterase inhibition.

The present study was designed to determine (1) the dose-response relationship between caffeine and mouse sperm motility; (2) the role of calcium in caffeine-regulated motility; (3) effect of caffeine on mouse fertilization in vitro.

SPERM CAPACITATION AND ACROSOMAL REACTION

Mammalian sperm, after completing transit through the epididymis, are still not able to fertilize oocytes. The final maturational process, called capacitation , was recognized 40 years ago by Austin (1951) and Chang (1951). Since then, capacitation has been studied extensively. However, the exact mechanism is still not well understood.

Sperm capacitation involves alterations in spermatozoan membrane composition and other characteristics (Ahuja, 1984; Langlais and Roberts, 1985; Wolf et al., 1986). The biochemical alterations of the sperm plasma membrane include removal or alteration of peripheral glycoproteins, rearrangement of integral glycoproteins, reduction in membrane cholesterol, and changes in the distribution and composition of certain membrane phospholipids (Yanagimachi, 1989).

The sperm membrane alteration was considered a process of removal of a decapacitation factor, which as a component of

epididymal fluid or seminal plasma stabilizes the sperm membrane preventing premature acrosomal loss. This component has been named, acrosome stabilizing factor (ASF) in rabbits (Thomas et al., 1986; Wilson and Oliphant, 1987) and Caltrin in bulls (Aumiller et al., 1988). In the mouse, there are two substances that conform to the traditional concept of a decapacitation factor, a-lactalbumin (Shur and Hall, 1982), and a low molecular weight proteinase inhibitor derived from seminal vesicle secretions (Aarons et al., 1984; Poirier et al., 1986).

In addition to the membrane alteration, various species-dependent modifications of spermatozoan accompany capacitation, including changes in metabolism (Hamner and Willians, 1963; Meizel an Turner, 1984; Neill and Olds-Clarke, 1988), Intracellular ionic composition (Working and Meizel, 1983; Mrsny, 1984; Babcock and Apfeiffer, 1987; Robbins and Boatman, 1988), acrosomal structure (Cummins and Yanagimachi, 1986; Dukelow and Williams, 1988) and adenylate cyclase activity (Monks and Fraser, 1987).

Stein and Fraser (1984) demonstrated that adenylate cyclase activity increases and phosphodiesterase activity decreases during capacitation of mouse spermatozoa in vitro. Such changes could provide increased availability of cAMP, which was demonstrated in hamster spermatozoa by White and Aitken (1989). That cyclic (cAMP) may play an important role

in sperm capacitation was proposed by some researchers (Tash and Means, 1983; Fraser and Ahuja, 1988; Fraser,1990). If this is true, caffeine, as a phosphodiesterase inhibitor, could increase the intracellular cAMP level and facilitate sperm capacitation.

Sperm maintain a low intracellular calcium concentration in the millimolar range, which is similar to other eukaryotic cells (Miller and Freedman, 1984; Miller, 1987). The maintenance of the concentration gradiance was believed to be by calcium pumping ATPase and a Na^{+}/Ca^{++} exchanger (Fraser, 1987). Any substances which interrupt the function of the calcium pump or the Na^{+}/Ca^{++} exchanger may cause an increase of the intracellular calcium concentration. A high level of calcium could lead to a toxic effect on sperm structure and function. This abnormal elevation of intracellular Ca^{++} may have a relationship to sperm capacitation and the acrosomal reaction.

The significance of capacitation was believed to encompass two separate aspects of sperm function: 1) the transition to hyperactivation, which allows the sperm to penetrate the cumulus oophorus and bind to the zona pellucida; and, 2) the development of the ability to undergo the acrosomal reaction (Bedford, 1990).

The sperm acrosomal reaction is an exocytotic process, with the formation of multiple fusion sites between the

plasma membrane and the outer acrosomal membrane in resulting the formation of vesicles and the subsequent release of the acrosomal contents. The major hydrolytic enzymes hyaluronidase and acrosin are involved in effecting sperm passage through the vestments surrounding the mammalian oocytes and other enzymes may be involved as well (Meizel, 1984).

There are two major factors responsible for the stimulation of the sperm acrosomal reaction. The first, is the egg-specific extracellular matrix component, ZP3 (Cherr et al., 1986; Philpott et al, 1987; Leyton and Saling, 1989;). The mouse zona pellucida glycoprotein ZP3 regulates acrosomal exocytosis by aggregating its corresponding receptors (ZP3-Rs) located in the sperm plasma membrane. These ZP3 receptors (Mr=95,000) also serve as substrate for a tyrosine kinase in response to zona pellucida binding (Leyton and Saling, 1989; Bunch et al., 1990). Florman et al. (1989) suggested that the zona pellucida induced the sperm acrosomal reaction by activating sperm membrane G protein, which triggered the elevation of intracellular calcium and pH and acrosomal exocytosis. Second, the acrosome reaction promoting factors, which are secreted by cumulus cells into the cumulus intercellular matrix (Meizel, 1985; Tesarik and Kopecny, 1986; Tesarik et al., 1988; and Siiteri et al., 1988) may be involved. One of these activities was originally ascribed to a protein or glycoprotein of apparent molecular weight of

approximately 50,000 (Suarez et al., 1986; Tesarik et al., 1988c) and recently believed to largely reside in protein-bound progesterone (Osman et al., 1989). Human follicular fluid is also considered a stimulator of sperm acrosomal reaction (Yudin et al.,1988; Mortimer and Camenzind, 1989; Stock et al, 1989). Within follicular fluid, there are many possible components which may be responsible for the reaction. However, progesterone is considered as the major stimulating element. In vitro studies have shown that 10 ng/ml to 1 ug/ml of progesterone significantly increase sperm intracellular Ca^{++} concentration and has a similar effect on stimulation of the sperm acrosome reaction (Thomas and Meizel, 1989; Blackmore et al., 1990). It has also been suggested that perhaps there is a coordinated or synergistic interaction between ZP3 and progesterone to initiate the acrosomal reaction (Blackmore et al., 1990).

These studies suggested a new mechanism which involved in steroid hormone action. The mechanism of progesterone action was probably mediated by a progesterone receptor resident in the plasma membrane of the sperm. Binding of progesterone to this receptor could activate a Ca^{++} channel in the plasma membrane, or the progesterone receptor may have inherent Ca^{++} channel activity. The phosphoinositide turnover and guanine nucleotide regulatory-binding proteins (G protein) do not appear to be involved in the progesterone induced Ca^{++} influx

response. One alternative is that the progesterone receptor activates phospholipase D, and that the increase in phosphatidic acid promotes Ca^{++} ionophoretic activity (Bocckino et al, 1987). A phospholipase D activity has recently been described in sea urchin sperm (Donino et al., 1989).

The methods for evaluating the sperm capacitation and acrosomal reaction have been developed and improved for many years. There are five techniques which are commonly used in different laboratories: 1. Transmission electron microscopy (TEM). It can provide micro-structural evidence for the acrosomal reaction (Yudin et al., 1988). 2. Phase-contrast and DIC microscopy. The acrosomal reaction of sperm with large acrosomes can be directly observed under phase-contrast and DIC microscope (Meizel et al., 1984). 3. Triple stain. It is the most commonly used technique, which has been applied for many species (Talbot and Chacon, 1981; Dudenhausen and Talbot, 1982; Varner et al., 1987; and Kusunoki et al., 1989). 4. Fluorescein isothiocyanate (FITC). With lectins or antibodies, FITC can demonstrate very good fluorescent intensity and contrast which provides an indirect image of the acrosomal reaction (Cross et al., 1986; 1989; Blach et al., 1989). 5. Chlortetracycline (CTC) assay. The method can provide a very fast result and has the unique potential of revealing the dynamics of the capacitation and acrosomal reaction (Ward and Storey, 1984; Lee et al., 1987; Endo et al., 1988). The CTC

assay was used in the present study.

DNA SPECIFIC FLUOROCHROMES

DNA specific fluorochrome (Hoechst 33258 and 33342) as non-toxic stains, have been widely used to measure cell density and proliferation (McCaffrey et al, 1988; Steuer et al, 1990), assess DNA distribution and synthesis (Latt and Stetten, 1976; Arndt-Jovin et al., 1977; Crister et al, 1983; Hinkley et al, 1986), observe sperm-egg fusion and preimplantation embryo development (Conover and Gwatkin, 1988; O'Rand et al, 1986; Wright and Longo, 1988; Sawicki and Mystkowska, 1990).

These bisbenzimidazole fluorochromes bind reversibly to sequences of three or more adenine-thymine pairs of double-stranded deoxyribonucleic acid (Comings et al, 1975; Richards et al, 1985). Binding appears to occur externally, rather than by intercalation.

The non-toxic properties of these dyes may depend on cell type and dye concentration. A study done by Conover and Gwatkin (1988) showed that mouse spermatozoa stained with 1 ug /ml Hoechst 33342 were capable of fertilizing mature oocytes,with resultant cleavage to the blastocyst stage, but 10 ug/ml of Hoechst 33342 significantly inhibited embryo cleavage. Loeffler and Ratner (1989) reported that 6 ug/ml of

Hoechst 33342 reduced lymphocyte motility and proliferative response. No data is available for cytotoxicity of Hoechst 33258 on mammalian gamete cells.

The mechanism of biological effect of DNA stains which interact with the DNA is thought to arise from the direct disturbance of the processes of DNA replication and transcription. (Smith et al, 1989).

REPRODUCTIVE TOXICITY OF TOLUENE

Toluene, also referred to as toluol, methylbenzene, methacide, and phenylmethane, is a volatile, aromatic hydrocarbon, commonly used in industry, and found in the home in spray paints, glues, and lacquers. The molecular structure is distinguished from that of benzene by the substitution of a methyl group for one hydrogen atom. Toluene has the molecular formula C_7H_8, a molecular weight of 92.13, a density of 0.867 at 20°C and a vapor pressure of 30 mmHg at 26 °C, (Kirk and Othmer,1963). Toluene is slightly soluble in water, 534.8 $\pm$ 4.9 mg/l (Sutton and Calder, 1975).

Toluene has been detected in municipal water supplies at levels ranging from 0.1 ug/l to 11 ug/l. The toluene metabolites benzaldehyde and benzoic acid were also found in municipal water at concentrations up to 19 ug/l (EPA,1980).

Animal reproduction experiments show impaired growth of

mother and fetus and fetal skeletal anomalies after exposure to large doses of toluene (Hudak and Ungvary, 1978 and Shigeta et al., 1982). Behavioral effects of toluene in mice exposed pre- and post-natally have been described (Kostas and Hotchin, 1981). Two children with multiple malformations were born to mothers who worked as shoemakers and were chronically exposed to toluene and trichlorethylene, used in a soling solution (Euler, 1967). There are a growing number of case reports on congenital defects in children born to mothers who had intentionally inhaled toluene in high doses throughout pregnancy. In one recent study, five women, who had previously borne children and were in their third trimester, developed severe renal tubular acidosis from paint sniffing and subsequently gave birth to five infants, three of whom were growth-retarded at birth, two showed craniofacial anomalies, and neonatal hyperchloremic acidosis (Goodwin, 1988).

Toluene has been shown to be teratogenic in one study by Nawrot and Staples (1979). Toluene was administered to CD-1 mice by gavage on days 6 through 15 of gestation at levels of 0.3, 0.5 and 1.0 ml/kg body wt/dose. A significant increase in embryonic lethality occurred at all dose levels and decreased fetal weight occurred at 0.5 or 1.0 ml/kg. In the 1.0 mg/kg group, a significant increase in the incidence of cleft palate was found which did not appear to be due merely to general retardation in growth rate.

Genotoxic effects of five widely used aromatic industrial solvents, ethylbenzene, methylbenzene (toluene), o-, m-, and p-dimethylbenzene (xylene), on bone marrow cells of male NMRI mice were studied using the micronucleus test (Mohtashamipur et al.,1985). Each compound was given to animals by IP administration of two similar doses 24 h apart. Increased formation of micronuclei within polychromatic erythrocytes of femoral bone marrow 30 h after the first injection was concluded to be due to the clastogenic effect of the test compound. Of the chemicals tested, only toluene gave a dose-dependent increase in the frequency of micronucleated polychromatic erythrocytes. This genotoxic activity of toluene was confirmed in male B6C3F1 mice.

An inhalation study done by Courtney et al.(1986) showed that toluene administered by inhalation at 400 ppm to CD-1 mice from days 6 to 16 of gestation was teratogenic but not fetotoxic. There was a significant shift in the fetal rib profile. At the lower concentration of 200 ppm, there was an increase in dilated renal pelves which might reflect desynchronization of maturation with respect to development and growth.

The cytogenetic effects of toluene are controversial. Forni et al. (1971) reported no evidence of chromosome aberrations in lymphocytes of toluene-exposed workers, and Gerner-Smidt and Friedrich (1978) reported no manifest sister-

chromatid exchanges following in vitro exposure of human lymphocytes to benzene, toluene or xylene. But reports from Bauchinger et al.(1983) and Schmid et al.(1985) indicated significantly increased sister-chromatid exchanges in man after exposure to toluene.

The limited studies on the teratogenic potential of toluene indicate that toluene could have some teratogenic and toxic effects on the mammalian fetus at high dosages with prolonged periods of dosing. No in vitro toxicological studies on the mammalian gamete cell after exposure to toluene were available. The present study is designed to test the effects of toluene on B6D2F1 mouse epididymal sperm motility and fertilizing ability in vitro.

MATERIALS AND METHODS

1. Experimental Animals

The animals used in this study were B6D2F1 mice. Originally all mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Since our own breeding program set up three years ago, this strain of mouse was produced at the Endocrine Research Center by crossing C57BL-6J and DBA-2J mice. B6D2F1 mice were sexed at twenty one days of age, and male and female mice were separated in different cages. Each cage contained four to five mice. They were provided Breeder Blox^{R} (Wayne Pet Food Division, Chicago, IL) and fresh water daily, ad libitum. Animal room light cycle was maintained on 12 h :12 h bases and temperature was maintained at 74-76 °C.

2. Culture Media

There were three market culture media used in this study. Brinster's BMOC-3 (Formula # 78-0012AG), TC-199 (# 320-1150AJ), and F-10 Nutrient Mixture (# 320-1550AG) were purchased from GIBCO BRL (Grand Island, NY). Medium M-16 was made according Whittingham (1971). The composition of these media is given in Table 1. The calcium-free BMOC-3 medium was made by modifying Brinster's BMOC-3 (pH=7.5$\pm$0.1; Osm=290$\pm$10).

All the culture media were held at 1-4°C for maximum of three weeks after opening the stock solution bottle. The medium was filtered with 0.22 um sterile filter unit (Milipore Products Division, Bedfard, MA).

Table 1. Comparison of the composition of four culture media (g/L)

Component*	BMOC-3	TC-199	F-10	M-16
NaCl	5.546	6.800	7.400	5.533
Na Lactate	2.253	-----	-----	2.610
Na Pyruvate	0.056	-----	0.110	0.036
NaH2PO4 H2O	-----	0.140	-----	-----
Na2HPO4 7H2O	-----	-----	0.290	-----
KCl	0.356	0.400	0.285	0.356
KH2PO4	0.162	-----	0.083	0.162
CaCl2	0.189	0.200	-----	-----
CaCl2 2H2O	-----	-----	0.044	0.252
MgSO3 7H2O	0.294	0.200	0.153	0.293
NaHCO3	2.106	2.200	1.200	2.101
B.S.A	5.000	4.000	4.000	4.000
Glucose	1.000	1.000	1.100	1.000

* The components of BMOC-3 was according to GIBCO Technical Services Department. The media TC-199 and F-10 were based on the GIBCO BRL Catalogue & Reference Guide, 1990. The medium M-16 was according to Whittingham (1971).

3. Sperm Motility

Mature male B6D2-F1 mice (3-6 month of age) were used. BMOC-3 culture medium was kept in the incubator overnight. Mice were sacrificed by cervical dislocation. One pair of caudae epididymides from each mouse was placed in 1.0 ml medium, which contained different concentrations of test chemicals. The epididymides were punctured with 22G1.5 Sterile needles allowing the sperm to swim into the medium. The sperm suspensions were further diluted to the final sperm concentration of 1-10 x 10^6/ml. Then 20 ul sperm suspension was placed on prewarmed hemocytometer and covered with a coverslip. The hemocytometer was under phase contrast Nikon microscope (Nikon, Garden City, NY) at 200X magnification. According to Schoff and Lardy (1987), and Berger and Beierle (1990), there are inherent inaccuracies in characterizing motility with regard to the degree of sperm vigor, I restricted my description of motility to percent of motile sperm. One hundred spermatozoa were counted every sample, the motility (%) was calculated based on the ratio of motile/immotile sperm. Each hemocytometer was counted twice and the average was used.

4. In Vitro Fertilization

Female mice, 4 weeks to 3 months of age, were induced to superovulate by i.p. injection of 8 i.u. pregnant mare's serum gonadotropin (PMSG) (Serotropin^{R}, Teizo, Tokyo, Japan), followed by 8 i.u. human chorionic gonadotropin (hCG) (Sigma, St. Louis, MO) 48 to 52 hr later. Twelve hr to 14 hr after hCG injection, two male mice were sacrificed, one epididymis from each mouse was collected and sperm collected as described above. The sperm suspensions were held at 37 °C, in a 5 % CO2 in air, humidified atmosphere. One hour after sperm incubation, female mice were sacrificed by cervical dislocation. Two oviducts with partial ovary and uterine tissue were collected from each mouse, and placed in 1.0 ml medium. The cumulus masses were recovered from the swollen ampulla and placed into 950 ul of medium with fine forceps. Each culture dish contained one to two cumulus masses and received 50 ul sperm suspension. The final sperm concentration was 1-10 x 10^{6}/ml. The mouse gametes were held at 37°C, in a 5 % CO_2 in air, 100% humidity for 24 hr.

In the experiment designed to measure the intracellular Ca^{++} change during fertilization, cumulus-free oocytes were used. The cumulus masses were dispersed by adding 30 ul of 10 mg/ml hyaluronidase (Sigma, St.Louis, MO) to 1.0 ml BMOC-3 medium. The final concentration of hyaluronidase was 300

ug/ml. After 5 minutes holding at 37°C, the oocytes were washed twice with BMOC-3 medium. After 24 hr incubation, assessment of fertilization was based on the presence of one of the following three criteria: (1). two or more polar bodies in the perivitelline space; (2). two or more equal size blastomeres. (3). In DNA staining experiments with Hoechst 33258, two or more pronuclei observed (Figure 1.)

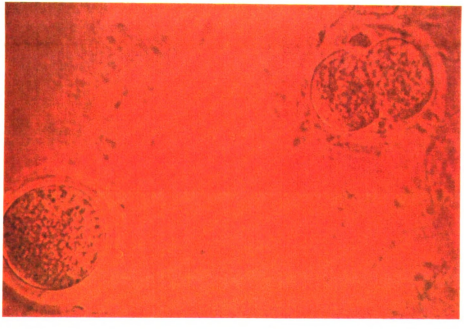


Figure 1. Uper right is a 2-cell stage mouse embryo and lower left is an unfertilized oocyte (400X).

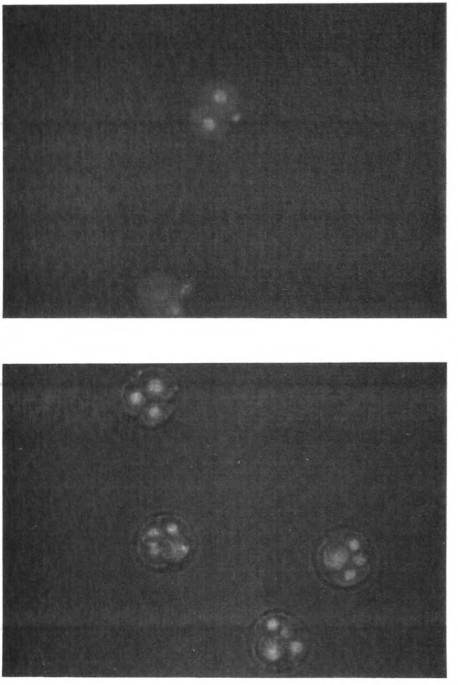


Figure 2. With Hoechst 33258, (A) One fertilized oocyte shows 2 polar bodies and another shows two nuclei (200X). (B) Four-cell mouse embryos.

5. CTC Assay for Capacitation and Acrosomal Reaction

The chlortetracycline (CTC) fluorescence assay described by Ward and Storey (1984) was used with slight modification. The experiment procedures were:

(1). Solutions: Buffer A for CTC was made of 0.3029 g Trizma Base; 0.455 g NaCl; 0.0788 g Cysteine and brought to 125 ml with double distilled water. CTC solution was made by adding 3.2 mg CTC (Sigma, St. Louis, MO) to 12.5 ml of Buffer A (500 uM). The pH was adjusted to 7.8. CTC solution was made fresh and kept in 0-4 °C through the experiment. Buffer B for Glutaraldehyde was made of 4.02 g Tris Hydrochloride; 2.97 g Trizma Base and brought to 50 ml with double distilled water (1M). 5% Glutaraldehyde solution was made by adding 3 ml of double distilled water; 3 ml Buffer B; and 1.5 ml 25% glutaraldehyde (Sigma) and filtered through a 0.22 um Corning sterile filter system (Corning Glass Works, #25932, Corning, NY). Buffer A and Buffer B were stored at 0-4 °C for a maximum period of three weeks.

(2). Procedure: Sacrificing mice and preparing sperm suspension have been described above. At 0, 30, 60, 90, and 120 minute time points during incubation, 10 ul of CTC solution was placed on a prewarmed (37°C) glass slide, immediately followed by 10 ul sperm suspension and mixed with a micropipette. The glass slide with the CTC-sperm suspension

was placed on a warming stage for 10 seconds and then fixed with 10 ul of glutaraldehyde solution, stirred thoroughly, and covered with a 24 x 50 mm cover glass. The slides were examined under a Nikon Optiphot microscope equipped with a fluorescence filter system: 380-425 nm excitation filter and 420 nm dichroic mirror (Figure 2.)

(3). Fluorescence patterns: The sperm with uniform bright fluorescence over head and midpiece were recorded as pattern F (fresh). Those with bright anterior head and dark posterior head were recorded pattern C (capacitated). The sperm with fluorescence lacking in the entire head but still in the midpiece were recorded as pattern A (acrosomal reacted) (Figure 3). One hundred sperm were scored on each slide, and the slides were examined within two hours of preparation.

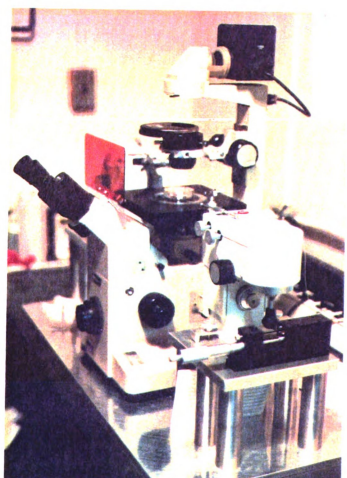


Figure 3. Nikon optiphot microscope equipped with a Leitz micromanipulator. The fluorescence system was not shown in the figure.

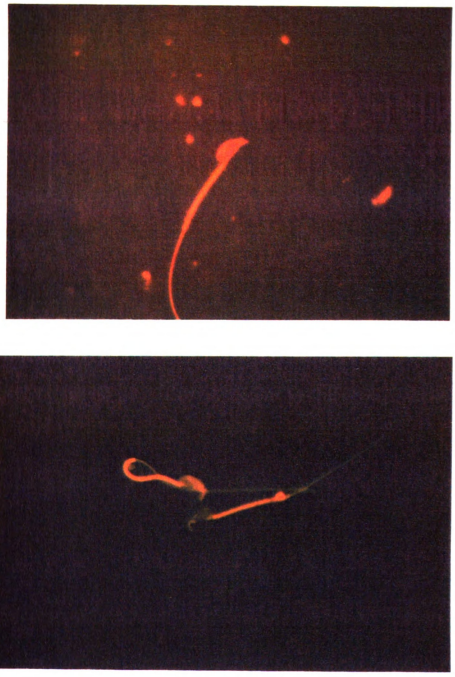


Figure 4. (A). One pattern F sperm showed the uniform bright fluorescence over head and midpiece. (B). The Sperm with bright anterior head and dark posterior head is pattern C sperm, and the sperm with fluorescence lacking in the entire head but still in the midpiece were recorded as pattern A sperm.

6. Intracellular Calcium Measurement

Single mouse sperm and oocyte intracellular Ca^{++} were measured by ACAS-470 & 570 Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI) (Figure 4.).

The mouse sperm suspensions were prepared as described above. Immediately after sperm suspension preparation, 5 ul of 1 mM (1.0 mg dissolved in 0.867 ml dry DMSO) Fluo-3 acetoxymethyl (AM) ester (Molecular Probes, Inc., Eugene, OR) was added per 1.0 ml sperm suspension. The final concentration of Fluo-3 AM was 5 uM. After 30 minutes incubation (37 °C, 5% CO_2 in air, 100% humidity), the sperm were washed twice in BMOC-3 by centrifugation at 2000rpm for 5 minutes at room temperature.

The oocyte collection was as described above. The oocytes with or without cumulus were incubated in 5 uM Fluo-3 AM for 30 minutes. The oocytes were then washed three times by transferring to fluorescein-free BMOC-3 medium with a micropipette.

Fluo-3 AM loaded spermatozoa and oocytes were transferred into a specially designed 35 mm culture dish (with a 0.15 mm thick coverslip on the bottom), which allowed using 100X oil objective. The sperm slide samples were also prepared for measuring intracellular Ca^{++} concentration over a time period. The dish or slide was observed under the inverted fluorescence microscope within ACAS-470 or ACAS-570. Fluo-3 AM can be

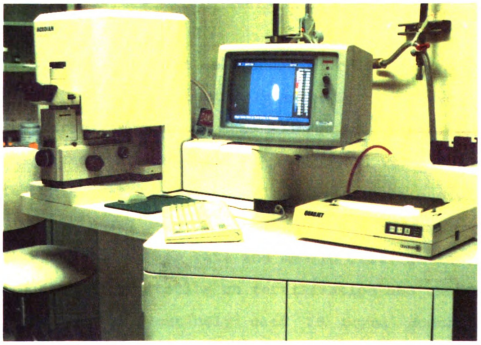
excited by the 488 nm line of the argon laser and emits in the visible spectrum similar to fluorescein (520 nm) (Wade, 1990). The laser excitation source provides a small beam (about 1 micron in diameter) which allows extremely low levels of fluorescence to be detected with minimum laser power. The X-Y scanning stage allows for 0.25 micron data acquisition to provide maximum spatial analysis. Two types of experimental data, the single sperm and oocyte integrated cross sectional line scans and two dimensional spatial scans, were collected and analyzed by computer software system.

The parameters used for measuring intracellular Ca^{++} level are shown in Table 2.

Table 2. The Parameters Used in Scan Image for Measuring Intracellular Ca^{++} within Single Sperm, Oocyte and Embryo.

Parameter*	Sperm	Oocyte	Embryo
PMT 1.	70-75 %	70-75 %	70-75 %
PMT 2.	10 %	10 %	10 %
Detector	1	1	1
Step Size	0.25 um	10 um	10 um
X Points	100-150	150-360	150-360
Y Points	100-150	150-360	150-360
Scan Str.	1-10 %	10-20 %	10-20 %
Laser Power	200 mW	200 mW	200 mW
Speed	20 mm/sec	5-10 mm/sec	5-10 mm/sec

* PMT 1 setting for the default detector in single detector mode, PMT 2 setting for the off-axis detector in dual detector mode. Step Size is the distance between data points. Speed means the maximum stage speed.



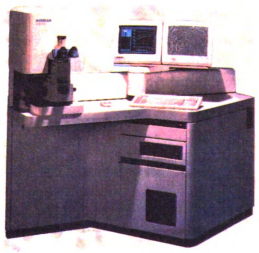


Figure 5. (A) ACAS 470 Interactive Laser Cytometer, (B) ACAS 570 Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI)

7. Mouse Gamete and Embryo Stain with Hoechst 33258

Two, four and eight cell mouse embryos were collected at 24, 48, and 60-72 hr respectively after in vitro insemination.

DNA-specific fluorochrome Hoechst 33258 (Aldrich Chem. Co., Milwaukee, WI; #86,140-5; F.W.=623.97) was dissolved and diluted in BMOC-3 medium. The final concentrations of 1, 10, 20, and 100 ug/ml were used to test the effects on sperm motility, fertilization rate and embryo cleavage in vitro.

To assess the results on fertilization and the stage of the embryo, We routinely used 10 ug/ml Hoechst 33258 incubated for 30 minutes. The embryos were then washed three times by transferring to dye-free medium. The dye-loaded embryos were observed under Nikon-Diaphot inverted microscope (Nikon, Garden City, NY) equipped with an epi-fluorescence filter combination UV-1A. The filter combination consisted of a 400 dichroic mirror, 365/10 excitation filter and a 400 barrier filter. The main wavelength for ultraviolet (UV) excitation was 365 nm (Roudebush, 1988).

8. Statistics

Different statistical tests were employed in different data forms. The contingency test (Chisquare test) was used in comparing fertilization rate and embryo degeneration rate. The Two-way ANOVA was used testing the difference of sperm CTC

pattern over time. Cochran's test was used to test homogeneity of variances. One-way analysis of variance (ANOVA) was used to test the difference of sperm motility with different concentrations of chemical treatment. After one-way ANOVA, the Student Newman Keuls (SNK) test was used for multiple group comparisons (Steel and Torrie, 1980). All P values less than 0.05 were considered statistically significant. The statistical software, MINITAB and SYSTAT were used to test these data.

RESULTS

I. Chemical effects on sperm motility

A. The effect of caffeine on mouse epididymal sperm motility in BMOC-3 medium

The mouse epididymal sperm motility in different concentrations of caffeine are shown in Table 3. No significant difference was found among four groups within 15 minutes, however, after 90 minutes and 4 hours incubation, the sperm motility in all three caffeine treated groups was significantly higher than the control group ($P < 0.05$). No dose dependence was found among three treatment groups.

B. The influence of Caffeine on mouse sperm motility in calcium-Free medium

This study (Table 4.) demonstrated that mouse sperm motility significantly decreased in the calcium-free medium after 90 minutes incubation ($P < 0.05$). Again there was no difference among three groups at 15 minutes. The results indicated that the calcium are required for sustained sperm motility. In the Ca^{++}-free medium, caffeine (10 mM) had no effect on sperm motility.

Table 3. The effect of caffeine on mouse epididymal sperm motility in BMOC-3 medium

Treatment	Sperm Motility (%)		
	15 min.	90 min.	4 Hr.
Control	73.4 ± 4.1 (8)	53.0 ± 2.1 (8)	31.2 ± 2.2 (8)
Caffeine: 0.1mM	79.8 ± 2.2 (3)	76.4 ± 3.6*(3)	60.7 ± 3.9*(4)
1.0mM	75.5 ± 4.8 (3)	76.4 ± 1.4*(3)	61.1 ± 5.5*(4)
10 mM	70.7 ± 13 (3)	74.5 ± 9.9*(3)	58.6 ± 4.4*(3)

Values are Mean ± S.E.M. * $P < 0.05$

Table 4. The influence of caffeine (10 mM) on mouse sperm motility in Ca^{++}-free BMOC-3 medium

Treatment	n	Sperm Motility (%)		
		15 min.	90 min.	4 hr.
Control	6	73.6 ± 8.2	52.6 ± 7.1	30.4 ± 4.3
Ca^{++}-Free	5	69.6 ± 3.2	31.4 ± 5.8 *	15.0 ± 4.8 *
Ca^{++}-Free (+10 mM Caffeine)	5	72.1 ± 6.2	30.4 ± 3.7 *	17.4 ± 2.9 *

* The difference compared to the control group was statistically significant ($P < 0.05$). No statistical significant difference was found between Ca^{++}-free and Ca^{++}-free plus 10 mM caffeine ($P > 0.05$).

C. The effect of DNA-specific fluorochrome (Hoechst 33258) on mouse epididymal sperm motility

The results of mouse sperm motility in BMOC-3 medium and treated with three concentrations of Hoechst 33258 are shown in Figure 6. There was no significant effect on sperm motility when the stain concentrations were 20 ug/ml or less. However, when the DNA stain concentration was 100 ug/ml, and after 60 to 90 minutes incubation, the sperm motility was significantly decreased ($P < 0.05$). Mouse sperm stained with 10 ug/ml Hoechst 33258 are shown in Figure 7.

D. The effect of toluene on mouse sperm motility in BMOC-3 medium

To test the effect of toluene on mouse sperm motility, four different concentrations of toluene, 0.0867, 0.867, 8.67, and 86.7 ug/ml were used. Because t-butanol was used as a vehicle conveying toluene into the medium, 1% of t-butanol was used as control group. The results are shown in Table 5. No difference was found between t-Butanol control and BMOC-3 control. The toluene concentration at 0.0867 ug/ml had no effect on sperm motility at all three time points ($P > 0.05$). However, when the concentration of toluene was higher than 0.867 ug/ml, the sperm motility was significantly decreased ($P < 0.05$).

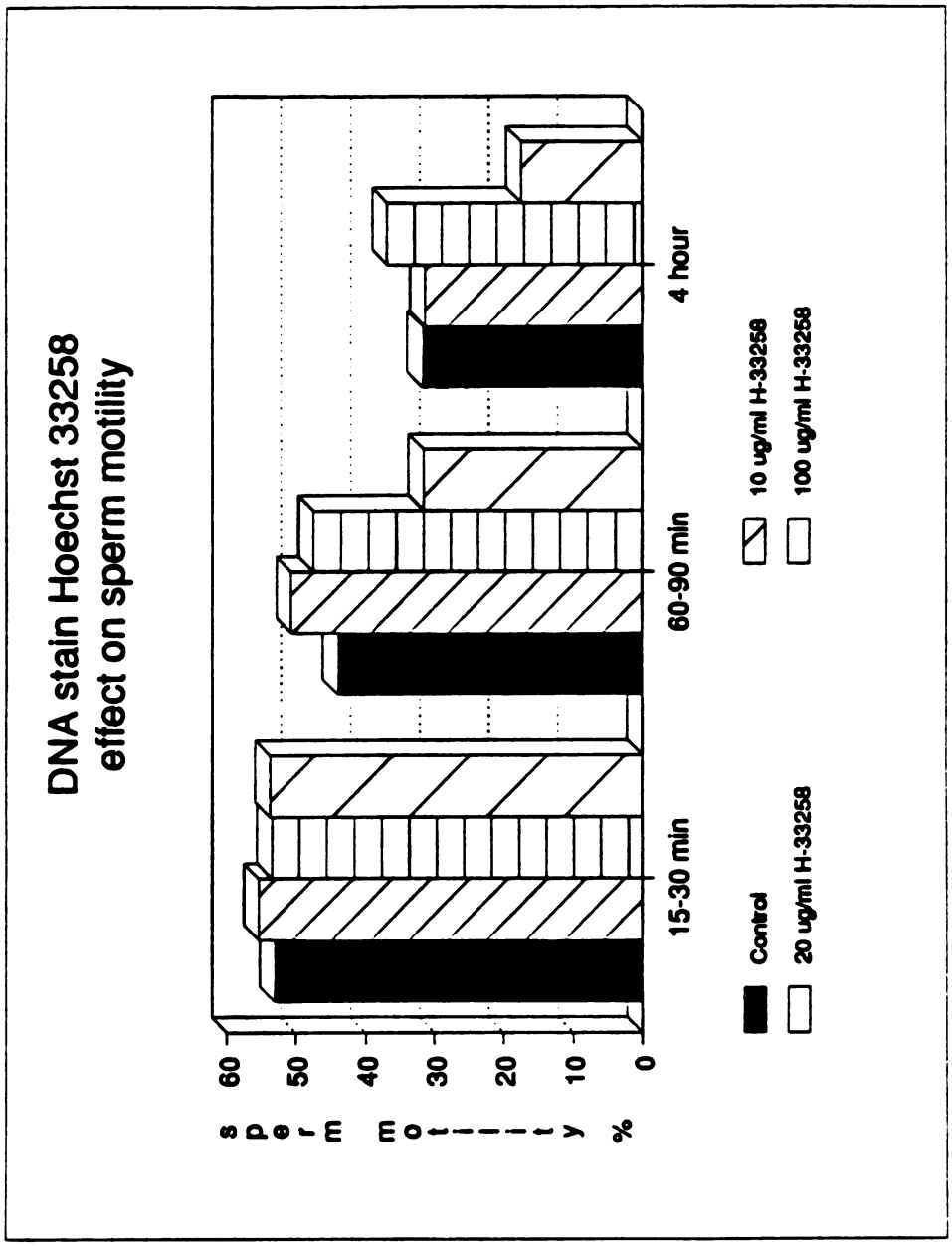


Figure 6. The effect of DNA stain Hoechst 33258 on mouse sperm motility in BMOC-3 medium.

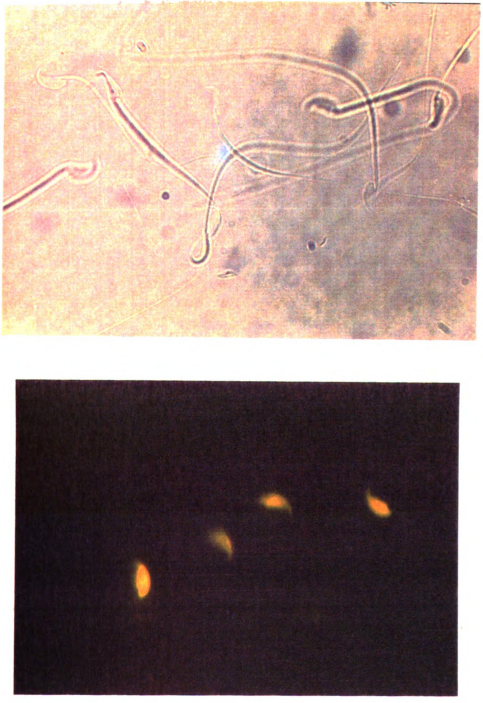


Figure 7. Mouse sperm stained with 10 ug/ml Hoechst 33258 for 30 minutes and under fluorescent microscope.

Table 5. Effect of toluene on mouse sperm motility in BMOC-3 medium during first 4 hour incubation

Group	% Motile Sperm		
	15-30 min.(n=8)	60-90 min.(n=5)	4 hr. (n=6)
Control A	74.2 ± 4.6	45.0 ± 4.2	28.0 ± 2.0
Control B	74.1 ± 3.9	50.0 ± 4.1	31.1 ± 7.9
0.0867ug/ml (T)	71.4 ± 6.4	45.4 ± 5.5	25.8 ± 5.3
0.867 ug/ml (T)	57.8 ± 6.7*	37.0 ± 3.4*	18.7 ± 4.3*
8.67 ug/ml (T)	46.9 ± 6.8*	27.9 ± 5.6*	17.3 ± 6.0*
86.7 ug/ml (T)	5.9 ± 5.7*	4.4 ± 6.2*	2.0 ± 4.5*

Control A is BMOC-3 medium and Control B is BMOC-3 medium with 1% of t-butanol; T = Toluene; Values are Mean ± SEM, * $P < 0.05$, statistically tested by One-way ANOVA and SNK.

II. Chemical effects on mouse sperm capacitation and acrosomal reaction

A. Caffeine effects on mouse sperm capacitation measured by CTC assay

The time courses of CTC patterns in the caffeine-free control group are shown in Figure 8. The results of effect of caffeine on mouse epididymal sperm capacitation are shown in Figure 9. During 2 hours period of incubation, in the control group, the percent of capacitated sperm (pattern C) increased from 14.4 % to 48.3 % (30 min.), 64.8 % (60 min.), then decreased to 41 % (90 min), and 23 % (120 min.); the caffeine treated group shows a similar change over time, However the difference between control group and caffeine treated group were not significant.

B. Caffeine effects on sperm acrosomal reaction during 2 hours incubation with BMOC-3 medium

The effect of caffeine on sperm acrosomal reaction is shown in Figure 10. In the control group, the percent of acrosomal reacted sperm (pattern A) were very low at 0 min. (8 %). It had no significant change during the first 60 minutes. The pattern A sperm were 8.25 and 12.8 % at 30 and 60 minutes respectively. However, the pattern A were very significantly increased at 90 minutes (41 %). They finally reached 71.6 % at

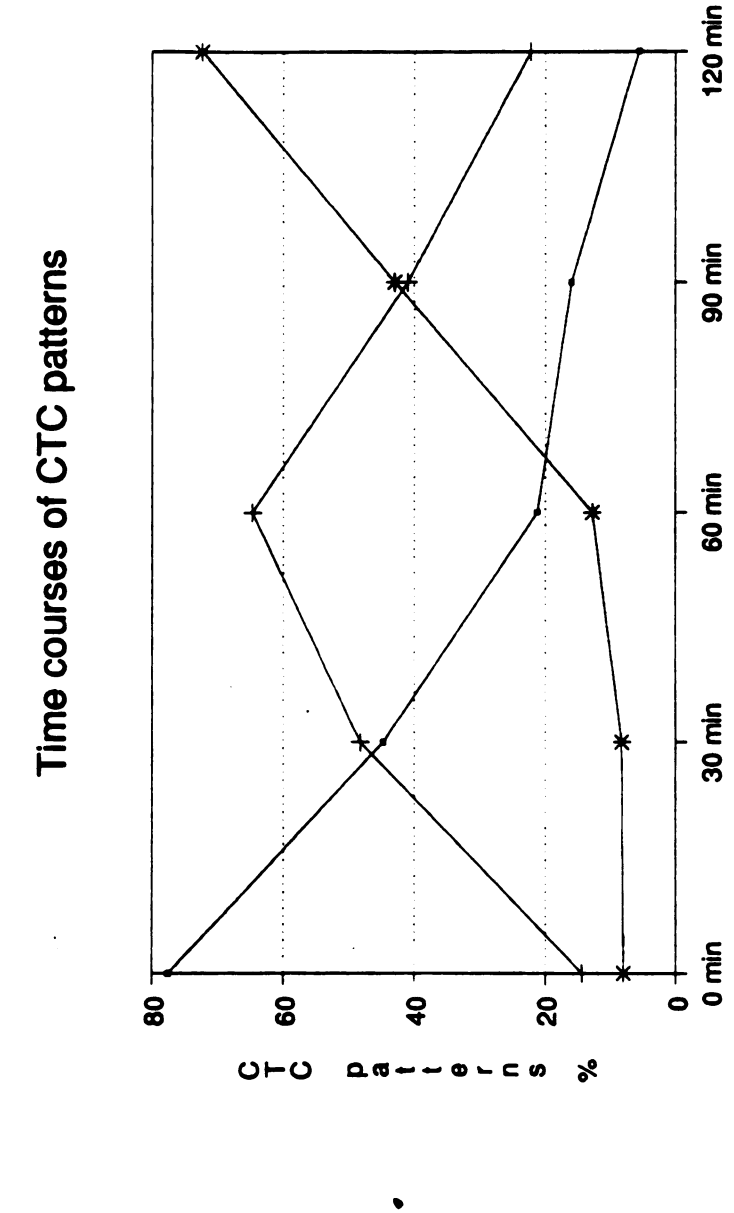


Figure 8. The time courses of mouse sperm CTC patterns in the caffeine-free BMOC-3 medium.

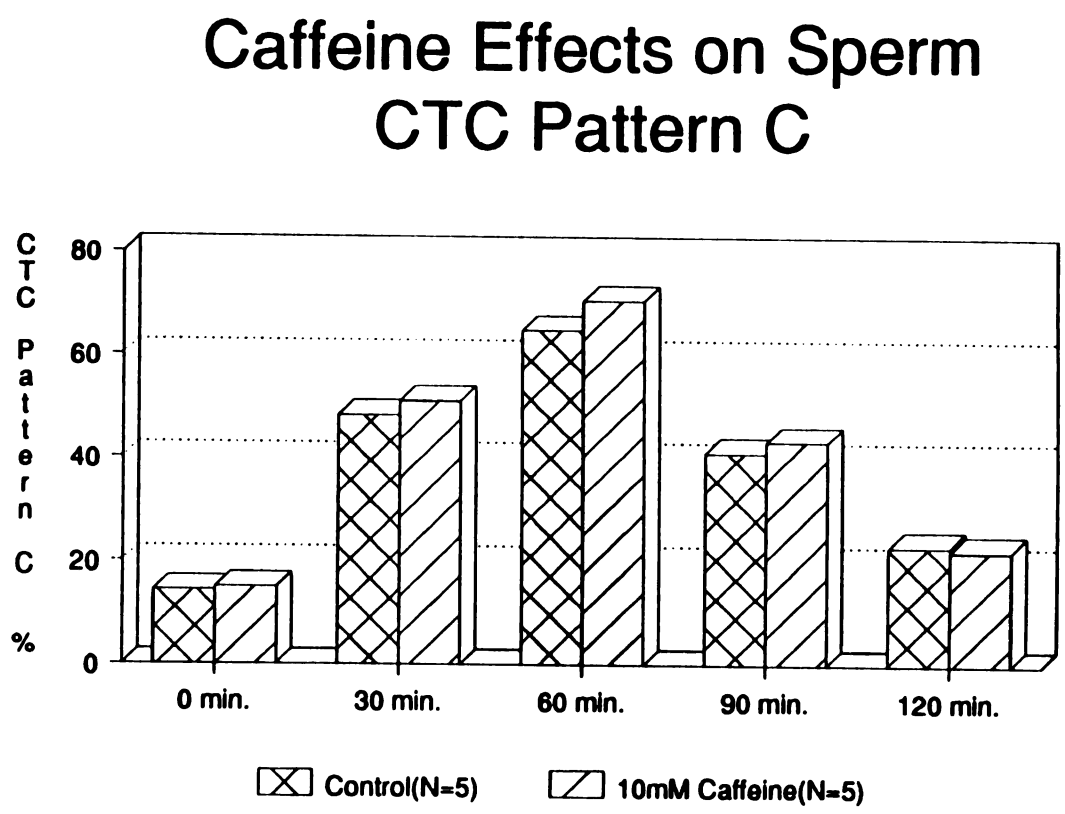


Figure 9. The influence of caffeine on mouse sperm capacitation (CTC pattern C) during 120 minutes incubation.

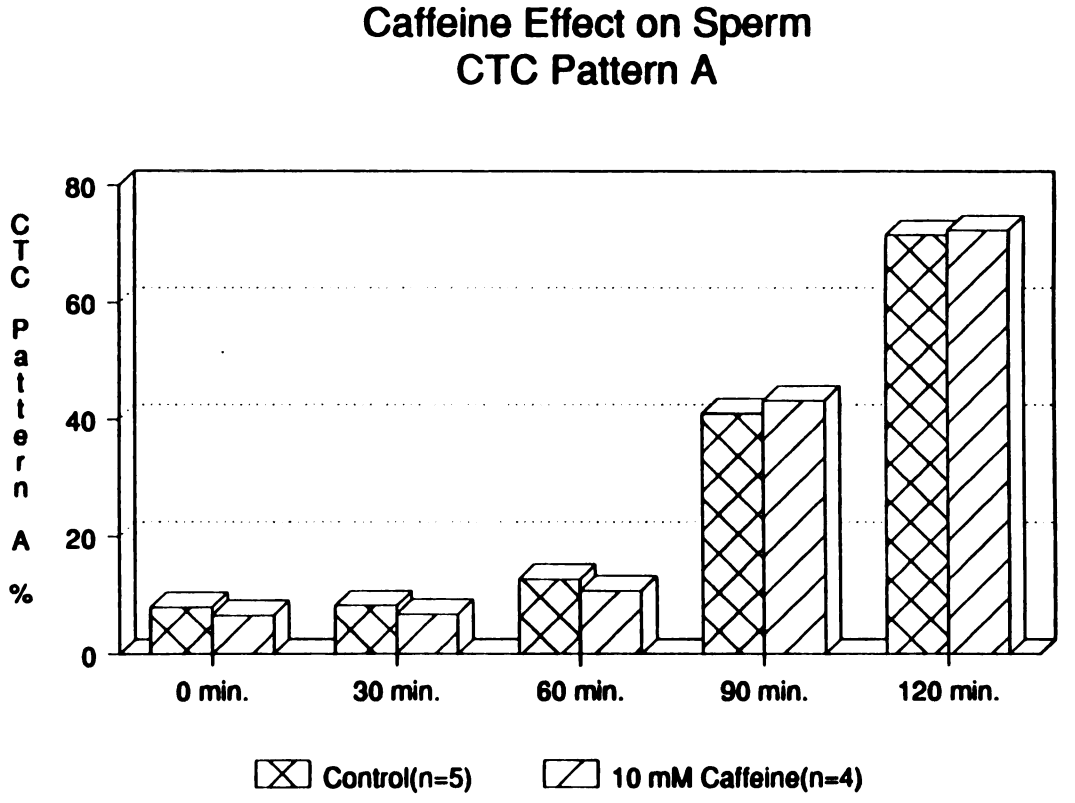


Figure 10. The effect of caffeine on mouse sperm acrosomal reaction (CTC pattern A) in vitro.

120 minutes. Comparing the control group, the caffeine (10 mM) treated group showed a similar change over time. No significant difference was found between the two groups ($P > 0.05$).

III. Chemical effects on in vitro fertilization

A. Culture media effects on in vitro fertilization

In order to determine the optimal medium for mouse in vitro fertilization, three common media were studied. The results are shown in Figure 11. There was no significant difference between BMOC-3 (69.7%) and M-16 (68.8%). The fertilization rate was significant lower in TC-199 (42.3%) ($P < 0.05$).

B. Caffeine effects on fertilization rate in vitro.

The effects of three concentrations of caffeine (0.1, 1.0, and 10 mM) on the fertilization rate are shown in Figure 12. Although a slight increase in fertility was observed with 0.1 mM caffeine, this difference was not significantly improved over controls. Caffeine (1.0 and 10 mM) decreased fertilization rate to 61.2% and 35.2% respectively, however the contingency test showed that only the 10 mM group was significantly different from control group ($P < 0.05$).

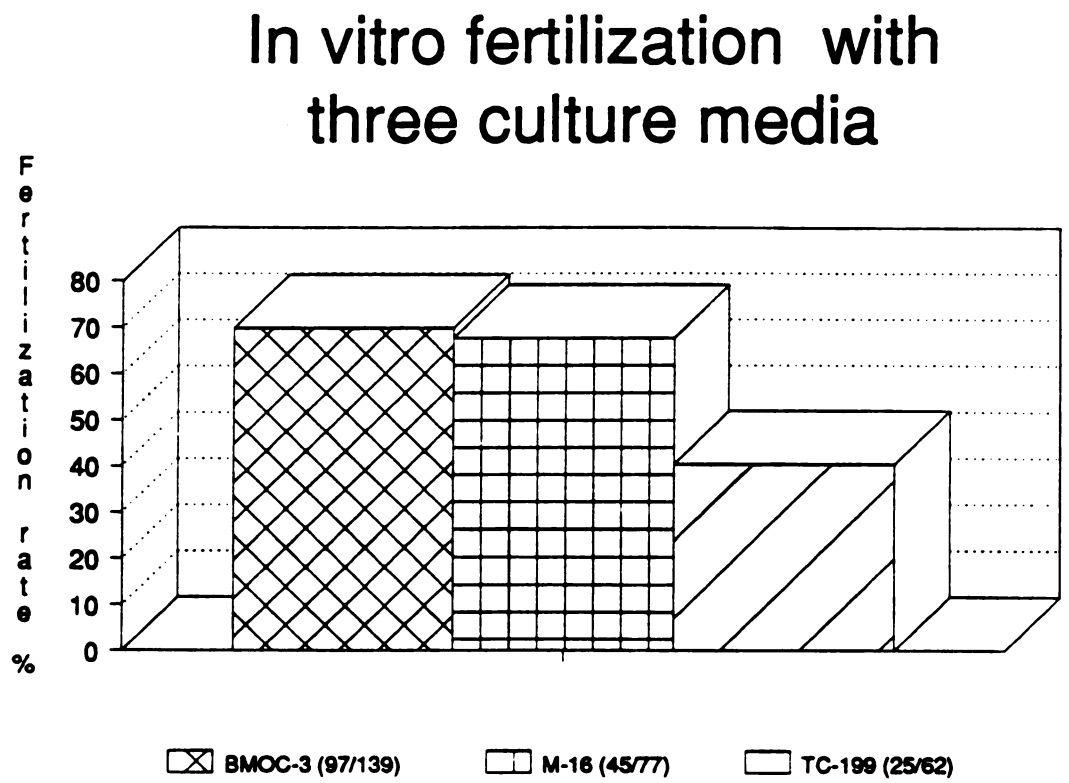


Figure 11. Mouse in vitro fertilization with three different culture media: BMOC-3, TC-199, and M-16.

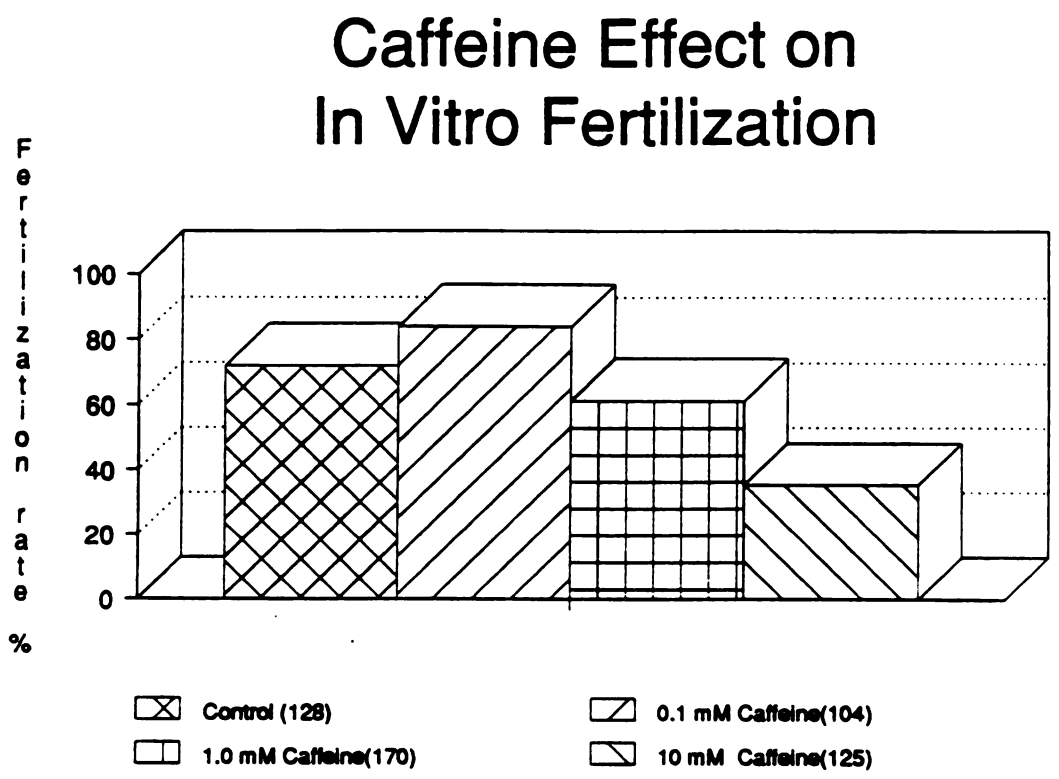


Figure 12. Effect of caffeine on mouse fertilization rate in vitro with BMOC-3 medium.

C. The influence of DNA stain Hoechst 33258 on In Vitro Fertilization

The experiment was designed to test the effect of Hoechst 33258 on in vitro fertilization. Both sperm and oocytes were exposed to same concentrations of Hoechst 33258 before insemination. The test concentrations of Hoechst 33258 were 1, 10, 20, and 100 ug/ml. The results are shown in Table 6. In stain-free control group, 169 oocytes were fertilized from 224 oocytes observed (75.5%). The gametes with 1.0 and 10 ug/ml of Hoechst 33258, had in vitro fertilization rates which were slightly lower than the control group, 72.3% and 66.7% respectively, but these values were not statistically significant ($P > 0.05$). The fertilization rates in the 20 and 100 ug/ml treated groups were much lower, 29.3% and 6.2% respectively, and were statistically significant ($P < 0.05$).

D. The effect of progesterone on in vitro fertilization rate

Recent studies have indicated that progesterone stimulates human spermatozoa acrosomal reaction (Thomas and Meizel, 1989). To test the possible effect of progesterone on mouse in vitro fertilization, the sperm and oocytes were incubated with varying concentrations of progesterone. The results are shown in Table 7. The fertilization rate in the control group was 93.2%, and the fertilization rate with progesterone (0.1, 1.0, and 10.0 ug/ml) treated groups were

93.5%, 89.9%, and 90.5% respectively. No statistical difference was found among these groups (P > 0.05).

Table 6. DNA-specific fluorochrome, Hoechst 33258 effect on mouse fertilization in vitro

Treatment	Fertilization Rate (%)	
Control	169/224	(75.5%)
1 ug/ml Hoechst 33258	133/184	(72.3%)
10 ug/ml Hoechst 33258	152/228	(66.7%)
20 ug/ml Hoechst 33258	41/140	(29.3%)*
100 ug/ml Hoechst 33258	10/162	(6.2%)*

* P<0.05, the differences were significant compared to the control group.

Table 7. Effect of progesterone on in vitro fertilization

Treatment	Fertilization Rate (%)
Control	68/73 (93.2%)
0.1 ug/ml progesterone	129/138 (93.5%)
1.0 ug/ml progesterone	62/69 (89.9%)
10 ug/ml progesterone	67/74 (90.5%)

No significant difference among groups (P>0.05).

E. Toxic effect of toluene on in vitro fertilization

The results of toluene on in vitro fertilization are shown in Table 8. No significant difference was found between two control groups ($P > 0.05$). No significant difference was found with the two toluene treated groups at the lowest level ($P > 0.05$). However, toluene concentration at the 8.67 and 86.7 ug/ml dose levels significantly inhibited in vitro fertilization, the fertilization rates were 32.9% and 6.3% respectively ($P < 0.05$).

Table 8. Toxic effect of toluene on mouse fertilization rate in vitro

Treatment	Fertilization Rate (%)
Control A (BMOC-3)	147/179 (82.1%)
Control B (t-Butanol)	125/163 (76.7%)
86.7ug/ml Toluene	5/80 (6.3 %)*
8.67ug/ml Toluene	24/73 (32.9%)*
0.867ug/ml Toluene	105/155 (67.7%)
0.0867ug/ml Toluene	130/164 (79.3%)

* $P<0.05$, the differences were significant compared to the control B.

IV. Chemical effects on mouse embryo development in vitro

A. DNA-specific fluorochrome and embryo development

Mouse gamete cells treated with DNA-specific fluorochrome Hoechst 33258 before in vitro insemination showed some effect on embryo development. The results are shown in Table 9. After 48-72 hours incubation, in stain-free control group, there were 61% mouse embryos developed from the two-cell stage to four or more cell stage. Although the 1 ug/ml Hoechst 33258 treated group had lower cleavage rate (48 %), the contingency test indicated that the difference was not significant ($P > 0.05$). When the stain concentration increased to 10 and 100 ug/ml, the embryonic development was significantly inhibited, the cleavage rate were 12% and 16.7% respectively ($P < 0.05$). It was also found that in additional limited trials at level of 20 and 100 ug/ml the number of degenerated mouse embryos increased (data not included). The degenerated mouse embryos are shown in Figure 13.

B. Toluene effects on oocyte and embryo degeneration after 24 hours incubation in vitro

The sperm and oocytes were cultured with different concentrations of toluene after in vitro insemination. After 24 hours incubation, the number of degenerated mouse oocytes and embryos was recorded each experiment. The results are shown in Table 10 and the degenerated mouse oocytes and

embryos are shown in Figure 14. Comparing the t-butanol control group, the degeneration rate was significant higher in 8.67 ug/ml ($P < 0.05$).

Table 9. Effect of DNA-specific fluorochrome Hoechst 33258 on mouse embryo development in vitro with BMOC-3 medium

Treatment	> 4 cell	2 cell	Embryo Cleavage (%)
Control	64	105	61.0 %
1 ug/ml	65	133	48.8 % (a)
10 ug/ml	6	50	12.0 % (b)
100ug/ml	2	12	16.7 % (c)

(a) Contingency test did not show significance ($P>0.05$)
(b), (c) The differences with control group were significant ($P<0.05$).

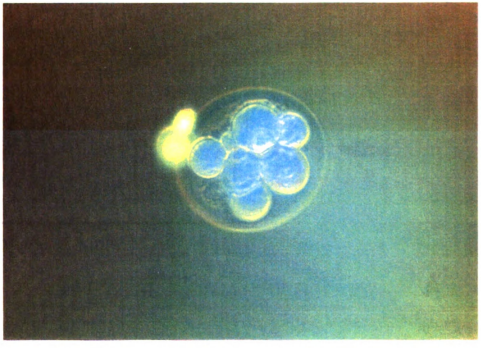


Figure 13. Degenerated mouse embryo after incubated with 100 ug/ml Hoechst 33258 for 24 hours.

Table 10. Influence of tolulene on mouse oocyte and embryo degeneration after 24 hours incubation in vitro

Treatment	No. of Degenerated	No. of Observed	Degeneration Rate (%)
Control	26	225	11.6 %
0.0867 ug/ml	21	215	9.8 % (a)
0.867 ug/ml	21	201	10.4 % (b)
8.67 ug/ml	46	137	33.5 % *

* Contingency test showed significant difference compared to the t-butanol control group ($P<0.05$). (a), (b) No difference was found compared to the control ($P>0.05$).

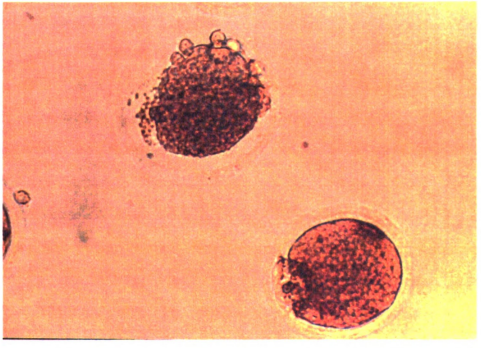


Figure 14. Degenerated mouse oocytes incubated with 8.67 ug/ml toluene for 24 hours.

V.Single gamete cell and embryo intracellular calcium measurement with ACAS 470-570 interactive laser cytometer

A. Single spermatozoa intracellular Ca^{++} measurement

1. Ca^{++} distribution in the sperm cell: Fluo-3 Patterns

To observe the sperm intracellular Ca^{++} concentration and distribution, two types of data were collected: integrated cross sectional line scan data and two dimensional spatial scan data. The typical cross sperm head line scan is shown in Figure 15. and the typical two dimensional spatial scan of sperm head and midpiece is shown in Figure 16. Different types of Ca^{++} fluorescent patterns were observed during the study. Pattern I. The sperm has very strong fluorescence within both head and midpiece, i.e. both head and midpiece contain high level of free Ca^{++}. This pattern of sperm is usually observed during first 30 minutes after preparation (Figure 17-A). Pattern II. The fluorescence in sperm head became very weak, the midpiece still has strong fluorescence (Figure 17-B). This pattern can be seen at any time, but the number of sperm showing this pattern increased after 30 minute. When the sperm head fluorescence became weak the fluorescence around sperm head did not change at all, i.e. the fluorescein still remained in sperm head, but the Ca^{++} level decreased. Pattern III. The fluorescent in sperm head disappeared, but the fluorescence in the midpiece remains the same (Figure 17-C). This pattern increases with time. There is a very high

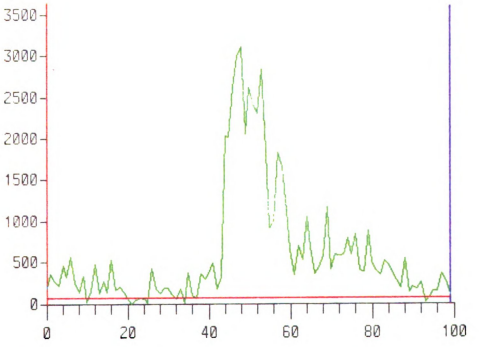


Figure 15. The typical integrated cross sperm head line scan

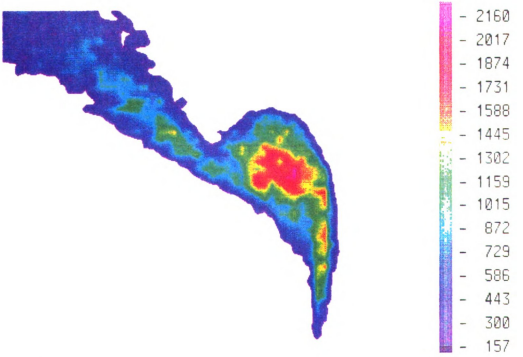
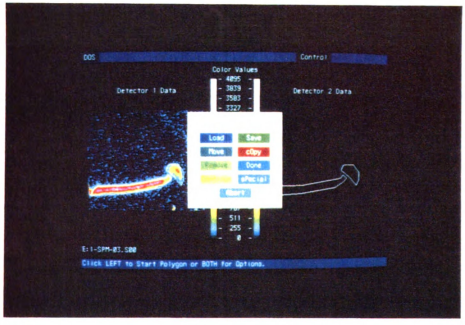


Figure 16. Two dimentional spatial scan of the sperm head and the midpiece



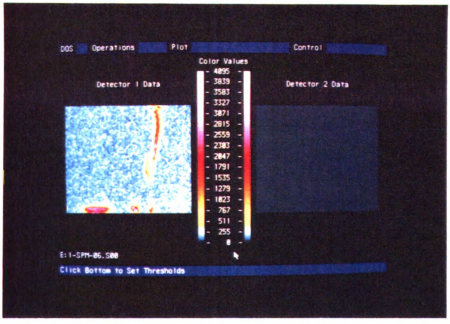


Figure 17. Fluo-3 density distribution: Ca^{++} fluorescence patterns (A) Pattern I sperm, (B) Pattern II sperm

percent of this pattern of sperm in the immotile group.

A relationship between the Ca^{++} fluorescence pattern and the CTC pattern was found in the study. The Ca^{++} fluorescence Pattern I was very similar to the CTC pattern F (fresh sperm); the pattern II appears similar to the CTC pattern C (capacitated sperm); and the pattern III was similar to CTC pattern A (acrosomal reacted sperm).

Because the sperm head and midpiece showed different Ca^{++} fluorescence distributions, and when the sperm head fluorescence disappeared the midpiece remained at a high level, this suggested that the Ca^{++} mobilization in sperm head and midpiece may involved different mechanism, and the Ca^{++} distribution in two compartments may be completely isolated. To test this hypothesis, I utilized a photobleaching technique. The Fluo-3 molecules within the sperm head were destroyed by a high power laser beam, then the fluorescence recovery over a period of time was observed. The results are shown in Figure 18. No recovery was observed within 14 minutes.

2. Chemical effects on single sperm intracellular Ca^{++} concentration

Single sperm intracellular Ca^{++} concentration was measured by the special fluorescent color values. The color values vary from 0 to 4095 specific color units, which were

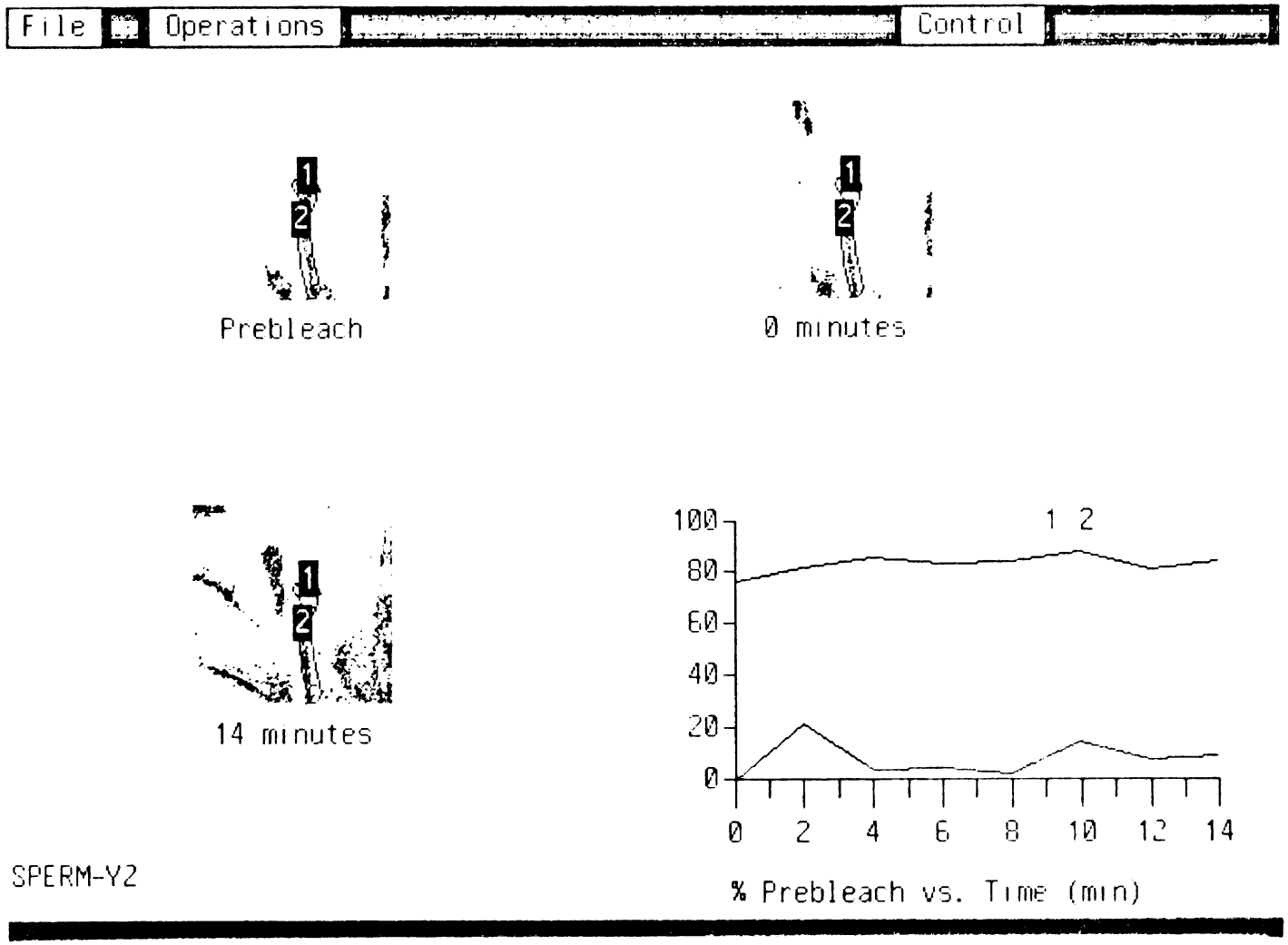


Figure 18. Photobleaching study of the sperm head fluo-3 recovery within 14 minutes

correlated with the Ca^{++} concentration. In this study, the relative fluorescence color value was used to detect the effects of chemicals on the intracellular Ca^{++} concentration.

a) Caffeine effects on sperm intracellular Ca^{++} concentration and distribution.

The image scan program was used to measure the caffeine effects on sperm intracellular calcium level. The results are shown in Figure 19. After adding 10 mM caffeine to the medium, a very significant intracellular Ca^{++} increase was observed in the sperm midpiece (the integrated value from 554,518 increased to 728,625), the change in the sperm head was not significant.

b) The influence of toluene on sperm intracellular Ca^{++} level.

The auto line scan program was used to measure the effect of toluene on sperm intracellular calcium concentration. The results are shown in Figure 20. In the sperm treated with 8.67 ug/ml of toluene, no significant calcium change was observed.

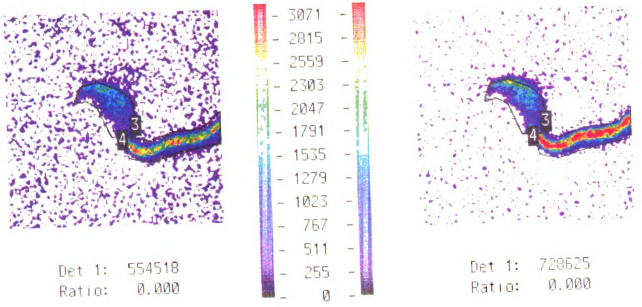


Figure 19. Caffeine effects on sperm intracellular Ca^{++} concentration and distribution

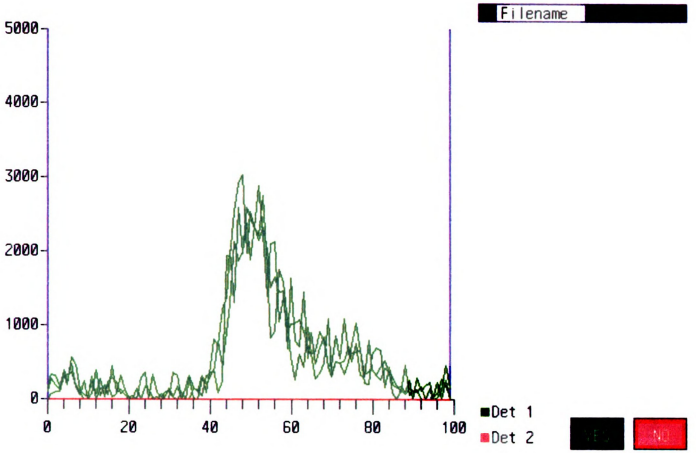


Figure 20. Toluene effects on sperm intracellular Ca^{++} measured by line scan

B. Intracellular Ca^{++} concentration in the oocytes

1. Ca^{++} distribution in mouse oocytes

The intracellular Ca^{++} distribution in mouse oocytes were measured by scan image program. Two types oocytes of were studied.

a) The fresh cumulus-intact oocyte

The Ca^{++} distribution in a cumulus-intact oocyte is shown in Figure 21. Because the Fluo-3 had to go through the cumulus cells, the fluorescence was very weak in the oocyte compared to the cumulus cells. After 5-10 minutes, the fluorescence became much stronger. There are two possible mechanisms involved. First, it could have occurred by passive diffusion from the medium to the zona pellucida or, secondly, it could have been by a cell-cell communication mechanism from the cumulus cells to zona pellucida through gap-junctions.

b) The cumulus-free oocyte

The intracellular Ca^{++} distribution in a cumulus-free oocyte (scan image) is shown in Figure 22, and the average fluoroscent intensity cross egg integrated value was 250,263 (Figure 23). The calcium concentration within the oocyte was different, at a low level in zona pellucida, an increase in the vitelline layer and plasma membrane, the highest in the cytoplasm. In the cytoplasm, there were some special areas showing very high Ca^{++} concentration. This was probably the

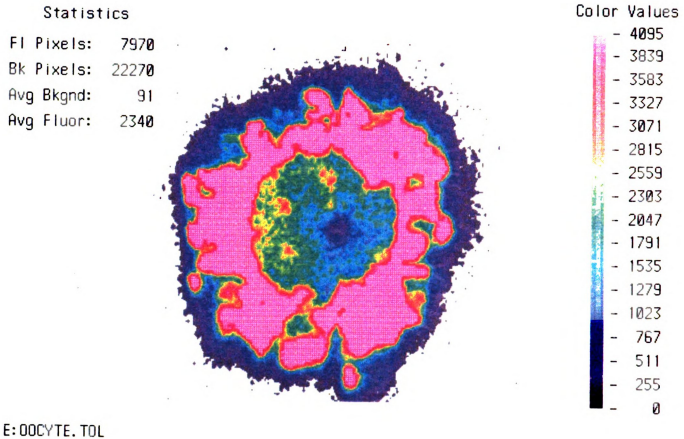


Figure 21. Fluo-3 density distribution in a mouse oocyte with cumulus cell

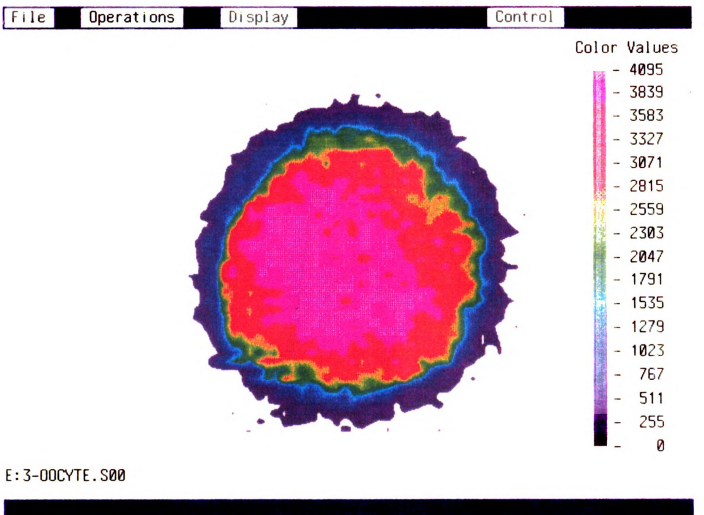


Figure 22. Cumulus-free mouse oocyte intracellular Ca^{++} distribution with image scan.

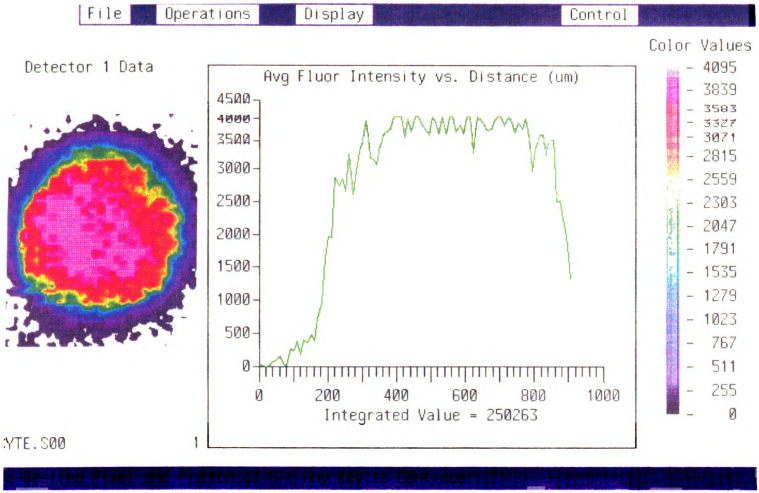


Figure 23. The average Fluo-3 fluorescence intensity of cumulus-free mouse oocyte.

intracellular free calcium storage, such as the endoplasmic reticulum or mitochondria. The oocyte 3-dimensional image of intracellular calcium distribution is shown in Figure 24.

2. Intracellular calcium change during sperm penetration

The intracellular Ca^{++} levels were measured in five mature oocytes during in vitro fertilization. The oocytes were treated with Fluo-3 but the sperm were not. After locating the oocytes, I used the line to record the basal calcium level, and then the sperm suspension was added to the dish. The sperm binding to the zona pellucida and penetration were observed by the video monitor. There was a continuously recorded intracellular Ca^{++} level in every oocyte by the line scan. After one sperm fused with the oocyte membrane, the line scan showed the calcium level significantly increased. The line scan before and after sperm penetration are shown in Figure 25. No significant calcium changes were observed in the rest of the oocytes.

C. Calcium distribution in the 2-cell embryo

Twenty-four hours after in vitro fertilization, 2-cell embryos were collected and treated with Fluo-3. The calcium distribution in the 2-cell stage embryo is shown in Figure 26. The calcium concentration in the two cells was not the same, one cell had a much higher calcium level than the other.

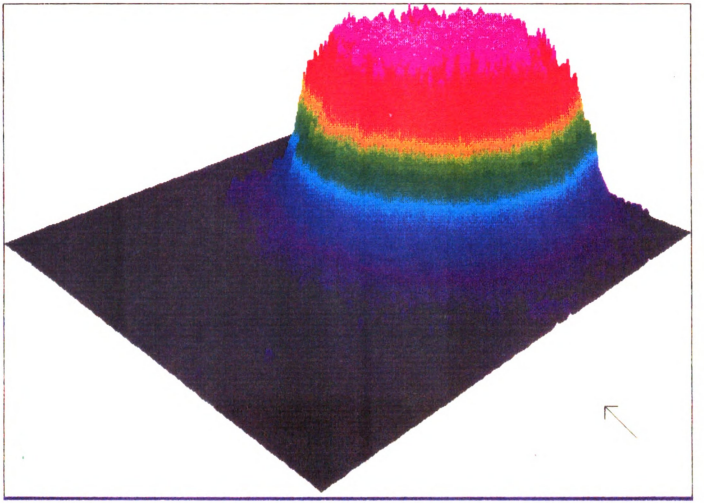


Figure 24. Three-dimensional image of mouse oocyte intracellular Ca^{++} distribution

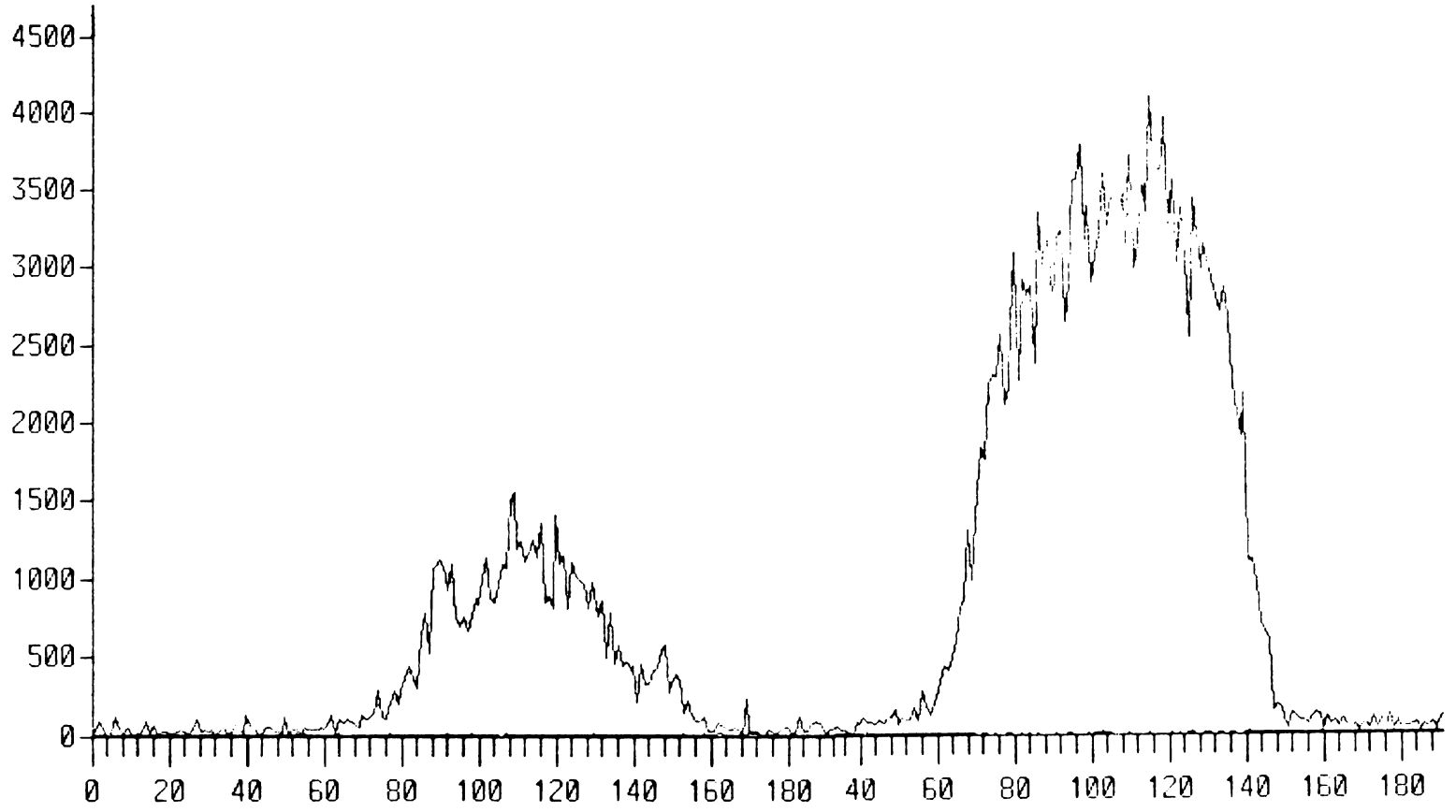


Figure 25. Mouse oocyte intracellular calcium change during sperm penetration

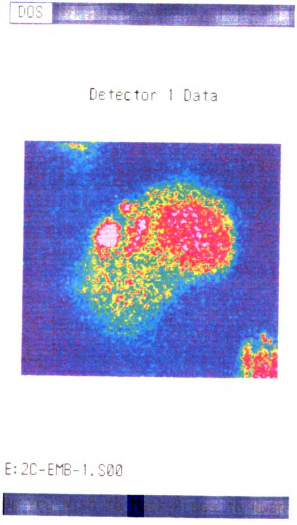


Figure 26. two-cell stage mouse embryo intracellular calcium distribution.

DISCUSSION

Chemicals and Sperm Motility

Sperm motility is a very important factor for a successful in vitro fertilization program. Mann and Lutwak-Mann (1981) indicated that there were clear correlations between sperm motility, fertilizing ability and pregnancy. Thus adverse chemical effects against the sperm can seriously effect reproduction, a simple method to test the possible effect of a chemical on in vitro fertilization is to test its effect on sperm motility.

Sperm motility is decreased after incubation for a period of time. Similarly, in vivo, the sperm motility significantly decreases after ejaculation. The reasons underlying the decrease in motility are not yet established. Two factors that are likely involved are the diminished integrity of the sperm membrane due to the proteolytic enzyme activity of the seminal fluid and /or the exhaustion of the sperm energy reserves (Huszar,1990).

We know that sperm motility is manifested by contractile waves in the sperm flagellum. The flagellar bending is produced by biochemical energy, mainly in the form of ATP resulting from oxidative phosphorylation, which is transferred into the biomechanical kinetic energy in the form of sliding

axonemal microtubules (Siegel et al., 1987). In mammalian sperm, cAMP was also proposed as a major factor in the initiation of flagellar movement and stimulation of motility during the fertilization process (Fraser, 1979; Chan 1983; Tash, 1989). Calcium and calmodulin also play a very important role in regulating sperm motility (Brokaw and Nagayama, 1985). There are many other factors e.g. sperm intracellular pH, Na^+, K^+ cation, and sperm membrane function, that are also involved in the modulation of sperm motility.

The effect of caffeine on sperm motility and velocity has been Known for twenty years. In most species, Caffeine stimulates sperm motility. The results from the present study showed that 0.1 to 10 mM of caffeine not only stimulated mouse epididymal sperm motility but also maintained a higher percentage of motile sperm for a longer period of time. The mechanism of caffeine stimulation of sperm motility is still not well understood. The classic explanation was that caffeine inhibited phosphodiesterase and increased the cellular cyclic AMP level (Hardman et al., 1971; Garbers et al., 1971). The stimulatory effect of cAMP on sperm motility has been confirmed by many studies (Lindemann, 1978; Mrsny and Meizel, 1980; Ishijima and Witman, 1985; Tash et al., 1986; Aaberg et al, 1989). Levin et al.(1981) analyzed the effects of caffeine on human sperm motility and cAMP phosphodiesterase. The results indicated that the direct action of caffeine on

sperm motility may not involve the inhibition of cAMP phosphodiesterase. The present study demonstrated that Ca^{++} was required for the caffeine stimulation of mouse sperm motility. In the Ca^{++}-free medium caffeine did not stimulate sperm motility. A single sperm intracellular Ca^{++} dynamic study showed that 5 mM caffeine increased mouse sperm midpiece Ca^{++} level, where the sperm mitochondria and axoneme are located. According to White and Aiken (1989), with a rise in intracellular calcium, and the resultant increase the adenylate cyclase activity. The rise in Ca^{++} may also mediated by calmodulin in the regulating sperm motility. This is probably another mechanism besides the inhibition of phosphodiesterase.

DNA-specific stain Hoechst 33258 is considered a non-toxic stain, which can bind to DNA and allows assessment of its organization and distribution within living cells. The present study demonstrated that a concentration of Hoechst 33258 of less than 20 ug/ml had no effect on sperm motility. When the concentration reached 100 ug/ml there was a decreased sperm motility after 90 minutes and 4 hours incubation. The mechanism for this effect is not clear. This high concentration of stain could cause an intracellular pH change or have a cytotoxic effect on the DNA structure and function.

Toluene has been detected in municipal water supplies at levels ranging from 0.1 to 11 ug/l. One in vivo study by

Nawrot and Staples (1979) indicated that there was a significant increase in embryonic lethality and decreased fetal weight following the oral gavage of pregnant CD-1 mice with toluene at 0.5 ml/kg level. There are no in vitro reproductive toxicological data available. In the present study, B6D2F1 mouse epididymal sperm were incubated with toluene from 0.0867 to 86.7 ug/ml for a 4 hour period. The results demonstrated that 0.0867 ug/ml of toluene had no effect on sperm motility. Concentrations of toluene greater than 0.867 ug/ml significantly decreased sperm motility at all three given times (15 min, 90 min, and 4 hr). The dose dependence was significant. The mechanism by which toluene inhibits sperm motility is not clear. Previous studies indicated that benzene significantly increased the intracellular calcium level by inhibiting the Ca^{++} pump. The high free Ca^{++} level was correlated with inhibition of protein phosphorylation and inhibition of sperm motility (Tash et al, 1988), but the single sperm intracellular calcium study reported here did not show a significant change after adding 8.67 ug/ml of toluene to the culture medium. It is interesting to note during the study of sperm intracellular Ca^{++}, when adding toluene to the culture medium, the sperm motility and vigor were very significantly increased, but after a few seconds the stimulation effect disappeared. It is not known why toluene had a short stimulation and long inhibition to the

sperm motility, or if toluene had an inhibitory effect on sperm mitochondrial oxidative phosphorylation.

Sperm capacitation and acrosomal reaction

During sperm capacitation, one of the principal events is a biochemical alteration of the sperm plasma membrane. (Cooper, 1986; Eddy, 1988; Yanagimachi, 1988). Such membrane alteration may be regulated by the sperm intracellular second messenger system. Stein and Fraser (1984) demonstrated that the adenylate cyclase activity increased and phosphodiesterase activity decreased during capacitation. Caffeine, as a phosphodiesterase inhibitor, could significantly increased intracellular cAMP level. The hypotheses of whether caffeine could change the sperm capacitation time course and/or trigger sperm acrosomal reaction has been suggested by the work of Kuehl and Dukelow (1982) and Chan (1983) on the squirrel monkey. The B6D2F1 mouse sperm was tested here by the CTC assay. The results did not show significant change of sperm capacitation time course nor acrosomal reaction. It could be the species difference or the lower sensitivity of CTC assay comparing to the triple stain. Sperm capacitation includes two significant changes, membrane alteration and sperm hyperactivation. The sperm hyperactivation was believed due to the membrane alteration in the sperm midpiece (Yanagimachi,

1989). The present study provided the new evidence of caffeine mobilizes sperm intracellular Ca^{++} in the midpiece region may involved in sperm hyperactivation.

The CTC assay as an indirect method for detecting sperm capacitation and acrosomal reaction has been used in many laboratories (Ward and Storey, 1984; Lee et al., 1987; Endo et al, 1988; and Kholkute et al, 1990). According to Ward and Storey (1984), the rational underlying this assay is that fresh epididymal sperm have CTC binding components absorbed to their plasma membrane surface and that these are lost during capacitation, so that the CTC binding pattern would change. An interesting finding from the present studies was that the CTC pattern had some correlation with sperm cell intracellular Ca^{++} distribution. Fresh sperm (Pattern F) had a high level of Ca^{++} both on the sperm head and midpiece; Capacitated sperm (Pattern C) had decreased sperm head Ca^{++} concentration; and acrosomal reacted sperm (Pattern A) had a Ca^{++} level that reached its lowest point in the sperm head but showed no change in the midpiece. This may provide some new evidences to explain the mechanism of the sperm CTC patterns.

In vitro fertilization

In the present study, four chemical substances were tested their effects on fertilization in vitro. The culture medium selection was based on a comparison of fertilization

rate with three culture media, BMOC-3, TC-199 and M16. The major component difference between high IVF rate media (BMOC-3 and M-16) and low IVF rate medium (TC-199) was sodium lactate and sodium pyruvate (Table 1), which were absent in the medium TC-199. According to a previous study in this laboratory (Chan, 1983), lactate and pyruvate are required to sustain sperm metabolism for the squirrel monkey with in vitro fertilization.

The effect of caffeine on fertilization was dependent on the concentration. Caffeine (0.1 mM and 1.0 mM) had no significant effect on the fertilization rate, although the percent of fertilized oocytes in the 0.1 mM caffeine treated group (83.9%) was higher than the caffeine-free control group (71.9%); and the 1.0 mM group was lower (61.2%). The caffeine at a concentration of 10 mM significantly decreased the fertilization rate (35.2%). This result was not in agreement with the study of Pomeroy et al. (1988), in which they demonstrated sperm pretreated with 6.0 mM caffeine increased the fertilization rate (62 %) comparing the caffeine-free group (23 %). Their control group had a very low fertilization rate probably because of different experimental procedures, in which they incubated the sperm with oocytes for only 15 minutes and used cumulus-free oocytes. The results from the present study suggested that the low concentration of caffeine (< 1.0 mM) may favor IVF; but the high level of caffeine (>

10 mM) was detrimental.

DNA-specific stains (Hoechst 33258 and 33342) have been used to detect early fertilization in many laboratories (Hinkley et al., 1986; O'Rand et al., 1986; Conover and Gwatkin, 1988; Roudebush 1988; Wright and Longo, 1988). No previous report is available on Hoechst 33258 toxic effects on gametes. The present study demonstrated that the mouse sperm and oocytes treated with 20 ug/ml and 100 ug/ml of the stain showed a significantly decreased fertilization rate. A concentration of Hoechst 33258 less than 10 ug/ml was considered a safe level. The mechanism by which the high concentration of this DNA stain affected fertilization is not clear. According to Smith et al. (1989) the biological effect of the DNA ligand (Hoechst 33258) on the cell may due to the direct disturbance of the processes of DNA replication and transcription.

It is not required for progesterone to be present for in vitro fertilization. The recent studies by Thomas and Meizel (1989) and Blackmore et al.(1990) have indicated that progesterone can cause an influx of extracellular Ca^{++} into the sperm and induce the acrosomal reaction. This suggested the hypothesis that the progesterone may enhance in vitro fertilization. The results from this study showed that media containing three concentrations of progesterone (0.1, 1.0 and 10 ug/ml) all had very good fertilization rates. However no

significant difference with controls was found.

The effect of toluene on fertilization was also dependent on the concentration. The critical toxic concentration of toluene was between 0.867 and 8.67 ug/ml. Since the toluene treatment involved both the sperm and the oocytes, the exact mechanism of this action is not known.

Embryonic development in vitro

Fertilization triggers the second meiotic division and extrusion of the second polar body. The critical elements of cellular proliferation and differentiation in mammalian embryonic development are influenced by many factors from either embryonic or maternal sources. Early embryo development is regulated primarily by the expression of specific genetic programs within the cells, but also requires a continuous supply of energy, hormones and growth factors, provided from maternal environment (Kaye, 1986). Chemical effects on embryonic development can occur at any stage, however the early stages are most easily influenced.

The results from the present study showed that DNA stain Hoechst 33258 (>10 ug/ml) significantly decreased embryonic cleavage. The possible mechanism has been discussed above.

The study also demonstrated that toluene at a concentration higher than 8.67 ug/ml significantly decreased

fertilization rate. The high concentration of toluene also increased the oocyte and embryo degeneration. One early in vivo study by Nawrot and Staples (1979) indicated that the pregnant CD-1 mouse receiving toluene at 0.3-1.0 ml/kg had significantly increased embryonic lethality. The results from this in vitro study provided the evidences that toluene could cause embryo death at early stage, and it may also suggested that higher level toluene exposure in the enviroment may cause fertility problems.

Intracellular Ca^{++} measurement in single gamete cells

Intracellular Ca^{++}, as a second messenger, plays an important role in regulating cell function. Because a Ca^{++} change is directly involved in sperm motility, capacitation, the acrosomal reaction, and sperm-egg fusion (Handrow et al., 1989; Stock and Fraser, 1989; Noland and Olson, 1989; Mortimer et al., 1988;), it is desirable to detect the single sperm cell intracellular Ca^{++} concentration, distribution and dynamic change during these events. Most of methods used measuring sperm Ca^{++} require millions of sperm (Babcock and Apfeiffer, 1987; Stephens et al., 1988;), and these methods lack sensitivity. The ACAS interactive laser cytometer makes it possible to measure intracellular Ca^{++} in a single living sperm or oocyte, and it also provides information on intracellular

Ca^{++} distributions.

Fluo-3 AM, as a new fluorescent Ca^{++} indicator with visible excitation and emission wavelengths, has been tested in many living cells (Joseph et al., 1989; Wade , 1989; Dawson and Hooper, 1990;). The binding of Ca^{++} to the fluo-3 increases the fluorescence up to 40 fold. The Ca^{++} dissociation constants are in the range of 0.37-2.3 um, and this gives better resolution than other indicators like Quin-2 or Fura-2 (Minta et al., 1989). Fluo-3 is useful for the determination of qualitative calcium changes in cells as the result of chemical treatment, however a major disadvantage of Fluo-3 is that it is difficult to analyze the absolute calcium levels (Wade, 1989). The present study used Fluo-3 as an indicator to determine the intracellular Ca^{++} distribution and dynamic change in the single sperm and oocyte. The results demonstrated that this fluorescein did give very good resolution for Ca^{++}.

In agreement with other studies (Endo et al., 1988), the results demonstrated that the calcium ionophore A 23187 (5 uM) significantly increased sperm intracellular calcium level within a few seconds. This is believed to be responsible for inducing the sperm acrosomal reaction (Tesarik, 1985; Lee et al., 1987; Anderson et al., 1989). A interesting finding from the caffeine treatment study was that caffeine (10 mM) also induced an intracellular Ca^{++} increase, but that the increase

was restricted to the midpiece of sperm. According to Vijayaraghavan et al. (1989), the plasma membrane surrounding the midpiece is considerably more permeable to calcium than the membrane domains surrounding the sperm head and tail.

The present study did not show a significant effect of toluene on sperm intracellular Ca^{++}. The toxical mechanism may not be interrupting the Ca^{++} metabolism, although other studies do show that benzene-type chemicals increase the intracellular Ca^{++} by inhibiting the Ca^{++} pump.

SUMMARY AND CONCLUSIONS

B6D2F1 mice were used for testing the effect of some physiological, pharmacological and toxic substances on reproduction. The in vitro study included testing the effects of chemicals on sperm motility, sperm capacitation and acrosomal reaction, intracellular Ca^{++} change, in vitro fertilization and embryonic development. This provided the following results:

(1) Caffeine (0.1, 1.0 and 10 mM) significantly increased mouse epididymal sperm motility. The stimulatory effect was Ca^{++}- dependent. Caffeine at the 0.1 and 1.0 mM levels had no significant effect on fertilization, but 10 mM of caffeine decreased the fertilization rate. The CTC assay demonstrated that caffeine (10 mM) did not effect sperm capacitation or acrosomal reaction.

(2) Fertilization rates were significantly decreased in the presence of 20 and 100 ug/ml of the DNA stain, Hoechst 33258. A concentration of Hoechst 33258 less than 10 ug/ml had no effect on fertilization, but gametes incubated with 10 ug/ml or higher levels of the DNA stain had inhibited embryonic cleavage.

(3) The inhibitory effect of toluene on mouse sperm motility was significant. The critical toxic concentration in vitro was between 0.867 and 8.67 ug/ml. A concentration of

toluene higher than 8.67 ug/ml not only decreased sperm motility and inhibited fertilization but also increased the percent of embryonic degeneration.

(4) Mouse sperm treated with caffeine, the intracellular Ca^{++} level significantly increased. But the Ca^{++} elevation was a slowly process and which only limited to the sperm midpiece. No significant effect of toluene on mouse sperm intracellular Ca^{++} change were observed.

In conclusion, the present study suggested that caffeine stimulation of mouse sperm motility may by mobilizing intracellular Ca^{++} distribution, especially increasing the mitochondria and axonemal Ca^{++} levels. The high concentration (> 10 mM) had an inhibitory effect on fertilization in vitro. The DNA stain Hoechst 33258 was reconsidered as a low-toxicity stain. A concentration less than 10 ug/ml was useful for these experiments. Toluene did have cytotoxic effect on gametes and embryos in vitro. These studies demonstrated that the sperm, oocyte and embryo intracellular Ca^{++} distribution patterns may provide some important information on gamete cell structure and function.

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APPENDIX

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