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Effects of Cyclophosphamide on Maturation and Subsequent Fertilizing Capacity of Pig Oocytes *in Vitro*

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Abstract

This study examines the effects of cyclophosphamide, a widely used anti-cancer agent, on the maturation of pig oocytes and on their subsequent fertilizing capacity in vitro. Pig cumulus-oocyte complexes collected from prepubertal gilts were cultured in Waymouth MB 752/1 medium supplemented with sodium pyruvate (50 μg/ml), luteinizing hormone (0.5 μg/ml), follicle-stimulating hormone (0.5 μg/ml), and 17β-estradiol (1 μg/ml) in the presence or absence of cyclophosphamide for 24 hr; they then were cultured without hormonal supplements in the presence or absence of cyclophosphamide for an additional 16-24 hr. The breakdown of germinal vesicle (GVBD) and changes in glutathione (GSH) content before in vitro fertilization were assessed. Oocytes containing one polar body and a metaphase plate were regarded as matured. Cytoplasmic maturation as determined by male pronuclear formation following fertilization in vitro was also examined. Treatment of oocytes with increasing concentrations (1-1000 μg/ml) of cyclophosphamide for 48 hr resulted in a dose-response inhibition of the rate of maturation, but had no effect on GVBD. Increasing duration (12-48 hr) of treatment with cyclophosphamide (100 μg/ml) led to a time-dependent inhibition of nuclear maturation, achieving statistical significance by 24 hr. The addition of cyclophosphamide (100 μg/ml) to maturation medium immediately after culture, 12 hr or 24 hr after culture also decreased the percentage of oocytes matured during a 48-h culture period. Exposure of oocytes to cyclophosphamide (100 μg/ml) for 40 hr did not prevent sperm penetration, not affect the incidence of polyspermy, or decrease the ability of oocytes to form a male pronucleus at 8 hr after insemination. The concentration of GSH, an important factor for male pronuclear formation, in pig oocytes was determined by an enzymatic cycling assay. The concentration found was 8.15±1.19 mM per oocyte. Exposure of oocytes to cyclophosphamide (100 μg/ml) had no effect on GSH concentration. These results demonstrate that cyclophosphamide directly inhibits the meiotic but not cytoplasmic maturation of pig oocytes *in vitro*. This inhibitory effect, apparently, is not mediated through a decrease in the level of intracellular glutathione.

Key Words: pig oocytes, cyclophosphamide, germinal vesicle breakdown, maturation, glutathione, fertilization

Introduction

An ovary in many mammalian species generally contains many follicles composed of various developmental stages. Shortly after birth, the ovary is populated by nongrowing primary follicles each containing a small (20 μm-diameter) oocyte arrested in the dictyate stage of meiosis and encircled by a single layer of granulosa cells. During its growth phase, oocyte diameter increases. Concomitant with increasing oocyte size, there is an increase in the number of granulosa cells surrounding the oocyte and enlargement of the follicle. Granulosa cells, apparently, play an important role in the regulation of oocyte growth (8). Just prior to formation of the follicular antrum the oocyte acquires the ability to resume meiotic maturation and undergo germinal vesicle breakdown. Oocytes removed from the

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follicular environment at this stage mature spontaneously in vitro. In vivo, fully-grown and competent oocytes are maintained in meiotic arrest until stimulated to resume meiotic maturation in response to gonadotropin surge. Although a large number of oocytes are present in ovaries (17), only a very small population of oocytes becomes available for ovulation, and most of the oocytes are lost at various stages of growth in the ovary.

Oocyte maturation is usually defined as those events associated with the initiation of germinal vesicle breakdown and completion of the first meiotic division, referred to as nuclear maturation. The ability of the mammalian oocyte to resume meiosis and complete nuclear maturation is acquired a stepwise fashion, which has been shown to increase with age of female (52) and size of follicle (58), though this ability differs among species studied (36). The ability of the oocyte to complete the first meiotic division is not evident until oocytes have nearly completed their growth phase and is associated with antrum formation (28). The process of oocyte maturation, however, also includes changes within the cytoplasm, of which the production of male pronuclear growth factor such as glutathione is a notable example (45).

Glutathione (γ-glutamylcysteinylglycine) is a major intracellular thiol that has important biological functions during cellular proliferation, amino acid transport, DNA and protein synthesis; and it protects cells against oxidation (32). The synthesis of glutathione during oocyte maturation appears to be a prerequisite for sperm chromatin decondensation and hence for male pronucleus formation after sperm penetration of mouse (10), hamster (45), and pig (62) oocytes. An inability by porcine oocytes to synthesize sufficient glutathione during maturation in vitro reduces their ability to form a male pronucleus after fertilization (62). The addition of cysteine to maturation medium increases the glutathione content of in vitro-matured porcine oocytes (63); whereas addition of low-molecular-weight thiols, such as cysteamine and β-mercaptoethanol, to culture medium enhances cysteine-mediated glutathione synthesis in in vitro-produced 6- to 8-cell bovine embryos (54) and increases synchronous pronuclear formation and normal embryonic development in porcine oocytes matured and fertilized in vitro (18).

Cyclophosphamide, a cytotoxic alkylating agent, has been commonly used in the treatment of cancer. Its cytotoxic effects are the result of chemically reactive metabolites that alkylating DNA and protein, producing cross-links (20). Consequently, the DNA or chromosomal damaging effects of cyclophosphamide have been reported widely in mammalian cells, both of somatic and germ cell origin (1, 48). In women, chemotherapy commonly produces menstrual irregularities, immediate or subsequent ovarian failure, and associated infertility (11, 26, 35). In pregnant mice administration of cyclophosphamide 60 h after copulation inhibits in vitro post-implantation development to blastocysts (25). When given 60 h after fertilization cyclophosphamide increases the number of nonviable blastocysts (24). While given orally to female mice in proestrus and estrus, cyclophosphamide causes a slight decrease in the number of implantation sites per animal and a significant increase in the percent dead embryos in comparison to controls evaluated on day 14 of pregnancy (30). In vitro studies on the effect of cyclophosphamide metabolites on fertilization and cleavage demonstrate a dose-related inhibition (5). These findings indicate that the oocytes are also potential targets for cyclophosphamide toxicity. Previous studies have also shown that postmeiotic germ cells are specifically sensitive to cyclophosphamide treatment (55-57). We hypothesize that cyclophosphamide exposure may affect the meiotic maturation of the oocyte. In this study, the effects of cyclophosphamide on oocyte maturation and on their subsequent fertilizing capacity were examined by using follicular oocyte-cumulus complexes isolated from prepubertal gilt ovaries as a model. Resumption of meiosis was assessed by the breakdown of germinal vesicle. The concentration of glutathione, monospermic penetration, and male pronuclear formation were then determined.

Materials and Methods

Culture Media

Basic medium for the manipulation of oocytes consisted of Waymouth MB752/1 (with L-glutamine but no sodium bicarbonate) supplemented with sodium pyruvate (50 μg /ml), fetal calf serum (5%), penicillin (100 U/ml), streptomycin sulfate (50 μg/ml), and fungizone (0.625 μg/ml) as described by Hirao et al. (21) with slight modifications. The maturation medium consisted of basic medium supplemented with 0.5 μg/ml porcine luteinizing hormone (USDA-pLH-B2), 0.5 μg/ml porcine follicle-stimulating hormone (USDA-pFSH-I1), and 1 μg/ml 17β-estradiol. The sperm preincubation medium (pH 7.8) consisted of modified Medium 199 (mM199; Earle's salts) containing calcium lactate (2.92 mM), sodium pyruvate (0.91 mM), glucose (3.05 mM), HEPES (25 mM), penicillin (50 U/ml), and streptomycin sulfate (50 μg/ml) and supplemented with 10% (v/v) fetal calf serum (16). The fertilization medium consisted of mM199 (pH 7.4) supplemented with 10 mM caffeine-sodium benzoate and 10% (v/v) fetal calf serum (16).

Collection of Oocytes

Pig ovaries were collected from prepubertal gilts at a slaughterhouse of Tai Yu Products Incorporated - Hsinying Factory and carried to the laboratory in 0.85% (w/v) NaCl containing penicillin (250 U/ml), streptomycin (250 μg/ml), fungizone (0.625 μg/ml), and sucrose (0.25 M) at room temperature. About 2 hr later, the follicular content was aspirated from medium-sized (3-6 mm in diameter) follicles with a 20-gauge needle and a 5-ml disposable syringe. Subsequently, the oocytes were separated and pooled using a stereomicroscope and washed twice in basic medium and once in maturation medium. Only oocytes surrounded by at least 3 to 4 uniform layers of compact cumulus cells were selected for the experiments.

In Vitro Maturation

Ten cumulus-oocyte complexes were transferred to a droplet of 100 μl of maturation medium covered with paraffin oil in a 60×15 mm dish, which was pregassed in a CO_2 incubator at 39°C for more than 3 hr, and then cultured at 39°C as described by Funahashi and Day (16) in an atmosphere of 5% CO_2 in air. After 24 hr of culture, the cumulus-oocyte complexes were removed from the medium and transferred to a pregassed droplet of 100 μl of basic medium after being washed three times in basic medium. The oocytes were then cultured for an additional 24 hr period at 39°C in an atmosphere of 5% CO_2 in air.

Sperm Preparation

The sperm-rich fraction of ejaculates was obtained from a Duroc boar by the gloved hand method at Taiwan Livestock Research Institute - Hsinhua. Semen samples were washed three times with 0.85% NaCl (w/v) containing 100 mg/L bovine serum albumin. Washed spermatozoa were diluted to 2×10^8 cells/ml in sperm preincubation medium (pH 7.8). The sperm suspension was incubated for 90 min at 39°C in an atmosphere of 5% CO_2 in air. The final sperm concentration used for insemination was 2×10^6 cells/ml.

In Vitro Fertilization

After 40 hr of culture, oocytes with an expanded cumulus mass were washed three times in fertilization medium and then transferred to a droplet of 50 μl of fertilization medium covered with paraffin oil, which was pregassed in a CO_2 incubator at 39°C for > 3 hr. The sperm concentration in preincubated medium (2×10^8 cells/ml) was diluted to 2×10^6 cells/ml with mM199 at pH 7.8 and supplemented with 10% fetal calf serum, and 50 μl of diluted spermatozoa were added to the 50 μl droplet containing oocytes. Oocytes were cultured with spermatozoa for 8 hr at 39°C in an atmosphere of 5% CO_2 in air.

Assessment for Maturation, Sperm Penetration, and Pronuclear Formation

At the end of the culture, oocytes were fixed for 48 hr with acetic alcohol (methanol and acetic acid, 3:1, v/v), stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and then examined under microscope with Nomaski interference optics. Resumption of meiosis was assessed by the breakdown of germinal vesicle. The meiotic stage of the oocytes was assessed according to Hunter and Polge (22). Oocytes containing one polar body and a metaphase plate were regarded as matured. Oocytes were designated as penetrated when they had at least one swollen sperm nucleus or a male pronucleus and corresponding sperm tail in the vitellus. Those oocytes with more than one swollen sperm nucleus or male pronucleus were considered to be polyspermic. Only oocytes containing male and female pronuclei with an intact nuclear membrane were considered as having formed male and female pronuclei.

Assay of Glutathione

After culture for maturation, cumulus cells were removed by agitation using a narrow-bore glass pipette in phosphate buffered saline (PBS; pH 7.4). Oocytes were washed three times with PBS. Groups of 25-30 oocytes in 5 μl PBS were transferred to a 1.5-ml microfuge tube and frozen at -20°C for 18 h. The frozen samples were thawed once, 5 μl of 1.25 M phosphoric acid were added to the tube, and then oocytes were ruptured by agitation using a narrow-bore glass pipette. The tubes containing samples were stored at -20°C until assayed. Blanks containing 5 μl of PBS without oocytes were similarly prepared.

Concentrations of glutathione in oocytes were determined by the DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]-GSSG (glutathione disulfide) reductase recycling assay (2). Briefly, 700 μl of 0.33 mg NADPH/ml in 0.2 M sodium phosphate buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μl of 6 mM DTNB in the stock buffer, and 190 μl of water were added into the microfuge tube containing thawed samples. Twenty five microliters of 100 IU/ml glutathione reductase were added with mixing to initiate the reaction. The rate of 5-thio-2-nitrobenzoic acid (TNB) formation was followed at 412 nm with a Beckman DU-640 spectrophotometer and was recorded at 0.5 and 2 min after the addition

of glutathione reductase. Both the assay reagent blank and glutathione standards (30-240 ng/200 μl) were assayed under the same conditions. The amount of glutathione in each sample was determined by comparison with a standard curve prepared at the same time. This amount was divided by the number of oocytes in the sample to obtain the total glutathione content per oocyte. The diameter of the oocyte was measured by means of inverted microscope with an ocular micrometer (0.01 mm). The mean cell volume was calculated by use of the equation $V = 4\ \pi r^3/3$ for the volume of a spherical body of known radius. Then, the glutathione concentration per oocyte estimated by the total glutathione content per oocyte and the mean cell volume was determined as described by Calvin et al. (10).

Statistical Analysis

Statistical analyses from two or four replicate trials for treatment comparisons were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA) program. All percentage data were subjected to arc sine transformation before statistical analysis. Oocyte glutathione levels were analyzed from eight replicates in 4 experiments by ANOVA via the STATVIEW program. Probability of $p < 0.05$ was considered to be statistically significant.

Results

Experiment 1

This experiment determined the effects of cyclophosphamide on maturation of pig oocytes *in vitro*. Oocytes were cultured for 48 hr in the presence or absence of various concentrations of cyclophosphamide. As shown in Table 1, treatment of oocytes with cyclophosphamide at concentrations of 1, 10, 100 and 1000 μg/ml for 48 hr resulted in a dose-response inhibition of the rate of maturation. Cyclophosphamide at all doses used did not prevent germinal vesicle breakdown (Table 1).

In the time-course experiment, oocytes were incubated for 12, 24, 36, and 48 h in the presence of 100 μg cyclophosphamide/ml, washed thoroughly and then incubated in the maturation medium without cyclophosphamide for an additional 36, 24, 12, and 0 hr, respectively. After 24 hr or longer of culture in cyclophosphamide, the rate of maturation was significantly reduced (Table 2). When cyclophosphamide was added to maturation medium immediately after culture or 12-36 hr after culture, the percentage of oocytes matured was also significantly reduced, achieving statistical significance by 24 hr (Table 3).

Table 1. Effect of Cyclophosphamide on *In Vitro* Maturation in Pig Oocytes[a]

Cyclophosphamide (μg/ml)	Number of oocytes examined	GVBD[b] (%)[d]	Matured[c] (%)[d]
0	157	132 (84)	111 (71)
1	159	139 (87)	91 (57)**
10	157	134 (85)	73 (47)***
100	159	135 (85)	37 (23)***
1000	154	135 (88)	28 (18)***

a Oocyte-cumulus complexes were cultured with various doses of cyclophosphamide for 48 hr.
b Germinal vesicle breakdown
c Oocytes with metaphase II chromatin and a polar body.
d Percentage of oocytes examined (mean of four independent experiments).
**$p < 0.01$, ***$p < 0.001$ when compared with control (cyclophosphamide = 0) value.

Experiment 2

This experiment was carried out to examine the effects of cyclophosphamide on in vitro maturation and fertilization of pig oocytes. Oocytes were cultured for 40 hr in maturation medium with or without cyclophosphamide (100 μg/ml) before oocytes were transferred to the fertilization medium. At 8 hr after insemination, 53% of the in vitro matured oocytes had been penetrated by spermatozoa and had already resumed meiotic progression from the metaphase II stage, and 64% had male pronuclear formation (Table 4). Exposure of oocytes to cyclophosphamide for 40 h did not prevent sperm penetration, not affect the incidence of polyspermy, or decrease the ability of oocytes to form a male pronucleus (Table 4).

Experiment 3

This experiment determined the effect of cyclophosphamide on intracellular concentration of glutathione, an important factor for male pronuclear formation, in pig oocytes. Cumulus enclosed oocytes were cultured for 48 hr with or without cyclophosphamide (100 μg/ml). The glutathione content in pig oocytes was assayed after cumulus removal. The mean diameter of a total of 234 oocytes was measured as 117.85×0.14 μm. The mean cell volume of pig oocyte was 857.01 picoliter as calculated by the equation $V = 4\pi r^3/3$, implying a glutathione concentration of 8.15±1.19 mM per oocyte (Fig. 1).

Table 2. Time Course for the Effect of Cyclophosphamide on *In Vitro* maturation of Pig Oocytes[a]

Time (hr) of culture in		Number of oocytes		
medium with cyclophosphamide	control medium	examined	GVBD[b] (%)[d]	Matured[c] (%)[d]
0	48	152	130 (86)	106 (70)
12	36	159	134 (84)	101 (64)
24	24	156	131 (84)	67 (43)**
36	12	157	133 (85)	54 (34)***
48	0	158	139 (88)	36 (23)***

a Oocyte-cumulus complexes were cultured with cyclophosphamide (100 Mg/ml) for 12, 24, 36, or 48 hr
b Germinal vesicle breakdown
c Oocytes with metaphase II chromatin and a polar body.
d Percentage of oocytes examined (mean of four independent experiments).
**p < 0.01, ***p < 0.001 vs. time of culture in control medium = 48 h.

Table 3. Effect of Cyclophosphamide Addition to Maturation Medium Immediately after Culture, or 12-36 h after Culture on *in Vitro* Maturation in Pig Oocytes[a]

Time (hr) of cyclophosphamide addition after culture	Number of oocytes examined	GVBD[b] (%)[d]	Matured[c] (%)[d]
Control	79	68 (86)	56 (71)
0	77	65 (88)	27 (35)***
12	78	66 (85)	29 (37)***
24	80	68 (85)	47 (59)**
36	77	63 (85)	49 (64)

a Oocyte-cumulus complexes were cultured for 48 hr in maturation medium with or without cyclophosphamide (100 μg/ml).
b Germinal vesicle breakdown.
c Oocytes with metaphase II chromatin and a polar body.
d Percentage of oocytes examined (mean of two independent experiments).
**p < 0.01, ***p < 0.001 vs. control

Exposure of oocytes to cyclophosphamide had no effect on glutathione concentration (Fig. 1).

Discussion

In the present study, cyclophosphamide was shown to elicit a dose- and time-dependent inhibition of the rate of maturation of pig oocytes *in vitro*. Cyclophosamide at concentrations used did not prevent germinal vesicle breakdown, indicating no influence on resumption of meiosis. The exposure of oocyte-cumulus complexes to cyclophosphamide had no effect on the ability of oocytes to form a male pronucleus at 8 hr after insemination. These results suggest that cyclophosphamide may not inhibit the cytoplasmic maturation of oocytes matured *in vitro*.

Cyclophosphamide is one of the most widely used anti-cancer agents. Koyama et al. (26) estimated that doses of 10.4, 9.3, or 5.2 g of cyclophosphamide resulted in amenorrhea in patients in their 20s, 30s, and 40s, respectively. In rodents, cyclophosphamide results in ovarian toxicity (4, 31, 34, 50). Cyclophosphamide preferentially damages larger follicles that are undergoing growth and induces acceleration of ovarian follicular loss (4). It has been demonstrated that granulosa cells are primary ovarian target cells for cyclophosphamide-induced ovarian toxicity (49). The oocyte is also a potential target of cyclophosphamide toxicity (5). The studies reported here provide additional evidence for the oocyte of target cell of cyclophosphamide by the demonstration of the inhibitory activity of cyclophosphamide on the rate of maturation of pig oocytes *in vitro*. It is generally recognized that cyclophosphamide shows mutagenic effect *in vitro* only after previous metabolic activation, normally present *in vitro* (27, 53). Cyclophosphamide is metabolized by the liver yielding highly reactive alkylating compounds. Therefore, cyclophosphamide activation *in vitro* requires enrichment of culture systems with liver enzymatic extracts. The ability of the ovary to activate cyclophosphamide-metabolizing capacity of rat *in vitro* has been studied on granulosa cells, rat ovarian tissue containing stroma, theca, and granulosa cells, and human granulosa cells (3). None of these tissues was capable of metabolizing cyclophosphamide to its active metabolites *in vitro*. However, in the present study we exposed cumulus-enclosed oocytes to cyclophosphamide without adding enzymatic extracts to the medium. Oocytes showed

Table 4. Effect of Cyclophosphamide on Serpm Penetration and Male Pronuclear Formation of Pig Oocytes Matured *in Vitro*[a]

Treatment	Number of oocytes examined	matured[b] (%)[e]	penetrated[c] (%)[f]	No. of polyspermic oocytes[a] (%)[g]	No. of oocytes with male and female pronuclei (%)[g]
Control	78	53 (68)	28 (53)	7 (25)	18 (64)
Cyclophosphamide	79	22 (28)***	13 (59)	4 (31)	8 (62)

a. Oocyte-cumulus complexes were cultured for 40 hr without (control) or with cyclophosphamide (100 μg/ml). All oocytes were fixed at 8 hr after insemination and then examined.
b. Oocytes with metaphase II chromatin and a polar body.
c. Oocytes had at least one swollen sperm nucleus and/or a male pronucleus and corresponding sperm tail in the vitellus.
d. Oocytes with more than one swollen sperm nucleus or male pronucleus.
e. Percentage of the oocytes examined (mean of two independent experiments).
f. Percentage of the oocytes matured (mean of two independent experiments).
g. Percentage of the oocytes penetrated (mean of two independent experiments).
***$p < 0.001$ vs. control.

their sensitivity to the presence of cyclophosphamide in the medium and the effect on maturation rate decreased as the concentration of the agent increased. Oocytes appear to maintain a structural and functional integrity, sufficient to react to cyclophosphamide without the addition of enzymatic extracts to the culture medium. These observations indicate that the mutagenic effect at the oocyte level can be investigated *in vitro* in the absence of enzymatic activation systems.

In this study a significant inhibitory action of cyclophosphamide on pig oocyte maturation was demonstrated when given immediately after culture for longer than 24 hr. The addition of cyclophosphamide late in the maturation process (i.e., before 24 hr of culture) also resulted in a remarkable decrease in maturation of the oocytes during a 48-hr culture period. Christmann et al. (12) reported that pig oocytes show heavy chromatin condensation at 12 hr after culture. By 24 hr most oocytes have entered metaphase I. Matured metaphase II oocytes were observed mainly after 35 h in culture and increased gradually to reach 85% of the total at 45-48 hr in culture (38). Therefore, the effect of cyclophosphamide on the maturation of oocyte appears to be time-dependent and is not due to acute toxicity. Furthermore, when oocytes cultured for 24 or 36 hr in medium with 100 μg/ml cyclophosphamide were washed and cultured in the control medium for an additional 24 or 12 hr, respectively, maturation of oocytes was still blocked. The *in vitro* effect of cyclophosphamide on oocyte maturation appears to be irreversible. It has been reported that cyclophosphamide treatment induced apoptosis of germ cells in rat testis *in vivo* (9). It is therefore possible that

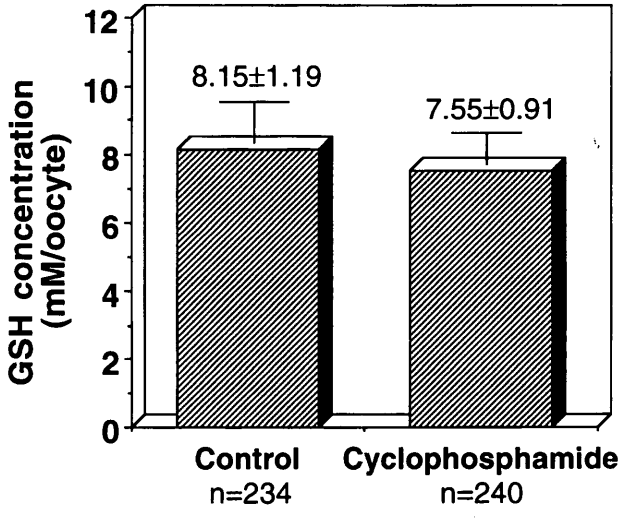

Fig.1. Effect of cyclophosphamide on glutathione (GSH) concentration in pig oocytes matured in vitro. Oocyte-cumulus complexes were cultured for 48 hr without (control) or with cyclophosphamide (100 μg/ml). The results represent the mean±SEM of 8 groups of oocytes cultured in 4 separate experiments. The total number of oocytes assayed is indicated as n.

cyclophosphamide may damage the oocytes which then undergo apoptosis, resulting in a reduction in the numbers of maturing oocytes. However, the ability of cyclophosphamide to induce apoptosis in oocytes remains to be confirmed.

The results of this study show that exposure of oocytes to cyclophosphamide before insemination does not prevent sperm penetration and not affect the incidence of polyspermic penetration. It is believed that the release of cortical granules during fertilization prevents further sperm penetration at the level of the

zona pellucida (zona reaction) and plasma membrane (vitelline block), although there are differences in the relative efficiencies of the zona reaction and of the vitelline block among species (61). For the pig it has been reported that the exocytosis of cortical granules occurs slowly and incompletely during *in vitro* fertilization (IVF) (13), resultimg in a high incidence of polypsermy after IVF (60, 64). Our findings suggest that this slow and incomplete exocytosis of cortical granules *in vitro* is apparently not affected markedly by cyclophosphamide treatment before IVF.

In the present study we found that cyclophosphamide did not affect the intracellular glutathione concentration of pig oocytes matured *in vitro*. Glutathione is an important cytoplasmic factor for sperm chromatin decondensation, and hence for male pronucleus formation following sperm penetration in pig oocytes (39). Yoshida et al (63) found that glutathione levels in pig oocytes change during oocyte maturation. In this study we also found that cyclophosphamide had no effect on the ability of pig oocytes to form a male pronucleus after IVF when oocytes were exposed to it for 40 hr of culture. These findings indicate that the effect of cyclophosphamide on nuclear maturation of pig oocytes is not mediated through a decrease in the level of intracellular glutathione.

It is of interest to consider the potential mechanisms involved in oocyte damage. Alkylating agents are known to have two important effects on intact mammalian cells: cell killing and delay in cell cycle progression with accumulation of living cells in the G_2 phase (33). The meiotic division in oocytes is a protracted process that is naturally arrested at the diplotene of the first prophase, which corresponds to the G_2 phase of the cell cycle. Meiotically arrested oocytes are referred to as immature oocytes. Resumption of meiosis in these oocytes is known as oocyte maturation and entails a G_2 to M transition. The transition from G_2 to M phase requires the cells to initiate complex processes including germinal vesicle breakdown, chromatin condensation, and reorganization of the cytoskeleton and progression to the metaphase II stage where they undergo a second arrest (41). The oocrrence of these events is regulated by a substantial increase in the oocyte's cytosolic kinase activity (14, 23). An important component of this activity is cyclin B-p34cdc2 kinase, also called maturation/metaphase-promoting factor (MPF) (7, 15). Active MPF is responsible for the onset of M phase in all eukaryotic cells including oocytes (37, 42). MPF is able to phosphorylate many of the proteins involved in nuclear membrane formation, chromatin condensation, and microtubular reorganization (19, 46, 59). In the present study, cyclophosphamide did not prevent germinal vesicle breakdown. These results indicate that cyclophosphamide does not influence resumption of meiosis. Since oocytes containing one polar body and a metaphase plate were regarded as matured, the observation that cyclophosphamide suppressed the rate of maturation of pig oocytes suggests that cyclophosphamide may prevent chromatin condensation or reorganization of the cytoskeleton. There is also some stereospecificity involved since cyclophosphamide does not inhibit overall maturation. It is reported that exposure to single high dose of cyclophosphamide produces DNA single strand breaks, as well as DNA-DNA and DNA-protein cross-links in postimplantation rat embryos (29, 47) and in testicular cells (51). Cyclophosphamide treatment can induce structural chromosomal aberrations and sister-chromatid exchanges in embryos (6), in Chinese hamster cells (40), in human chorionic villi (44), and in germ cells at various stages of spermatogenesis (43). It is therefore possible that cyclophosphamide induces these defects during culture of oocytes *in vitro*. These cytogenetic effects probably result from cyclophosphamide-induced cross-linking of DNA. On the other hand, cyclophosphamide may also alkylate other macromolecules, such as proteins and enzymes, that are involved in chromatin condensation and microtubular reorganization. As MPF is involved in both nuclear membrane breakdown and chromosome condensation during M-phase, cyclophosphamide may affect its activity. The levels of both MPF components, i.e., $p34^{cdc2}$ and cyclin B, as well as its activity in pig oocytes cultured *in vitro* with cyclophosphamide remain to be determined.

In conclusion, our results indicate that cyclophosphamide inhibited nuclear maturation of pig oocytes *in vitro*. Additional studies are necessary to clarify the mechanism involved in the suppressing effect on the maturation of pig oocytes.

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