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Original Article

Combined treatment with cysteamine and leukemia inhibitory factor promotes guinea pig oocyte meiosis *in vitro*

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Abstract: The guinea pig is an excellent but underused animal model due to its reproductive biology, which poses difficulties in inducing superovulation, embryo manipulation *in vitro*, and embryo transfer. We examined the effects of cysteamine (Cys), leukemia inhibitory factor (LIF), and Y27632 on guinea pig oocyte *in vitro* maturation (IVM). Cumulus-oocyte complexes were collected from antral follicles and classified into three different types before IVM. Among type I oocytes, maturation rates to metaphase II (MII) were similar in basal maturation medium and medium supplemented with Cys or LIF (39.5-40.9%), but combined Cys and LIF treatment increased the MII rate to 61.8%. Supplementation with Y27632 alone or in combination with Cys and LIF dramatically reduced the MII rate (27.7-29.7%). Similar trends were observed for type II oocytes, although their overall MII rate was lower than that of type I oocytes. The MII rate was higher among oocytes collected from 2-month-old guinea pigs compared with those from 4-month-old guinea pigs (56.5 vs. 44.8%). The optimal IVM duration was 24 h (52.5%), as 36 or 48 h of IVM reduced the MII rate (32.8-42.5%). Furthermore, Y27632 reduced the presence of microfilaments in oocytes. These findings indicate that combined supplementation of maturation medium with Cys and LIF, but not Y27632, improves the maturation efficiency of guinea pig oocytes. This study provides an important scientific basis for further efforts toward guinea pig *in vitro* fertilization, cloning, and gene editing by establishing an animal model for human reproduction and related diseases.

Keywords: Guinea pig, oocyte, maturation, meiosis, F-actin

Introduction

The guinea pig (*Cavia porcellus*) is an excellent model for studying human reproduction and implantation as well as depression, immunity, and respiratory diseases [1]. Distinct from other experimental rodents, guinea pigs have a longer estrus cycle (16 days) and gestation duration with fewer ovulations [1-3]. Guinea pigs generally exhibit a complete estrus cycle including follicular and luteal phases, similar to humans, pigs, cattle, and sheep [4]. During a guinea pig estrus cycle, ovarian follicles undergo two stages with follicular waves peaking at 10-11 d and 16 d, similar to cattle and sheep [5, 6]. In addition, guinea pig blastocysts undergo similar interstitial implantation as human blastocysts and form syncytiotrophoblast cells [7, 8]. However, the use of guinea pigs as an animal model in reproduction studies is challenging, as they have only 3.8 ovulations per ovulation cycle [3], and conventional hormones (e.g., pregnant mare's serum gonadotropin, human chorionic gonadotropin) do not successfully induce superovulation [9-11]. Although several approaches have been employed to stimulate ovulation in guinea pigs, including inhibitory alpha-inhibin active immunity [12],

equine chorionic gonadotropin [13], altrenogest [1], and human menopausal gonadotropin treatment [5], they have achieved little success.

The *in vitro* maturation (IVM) of oocytes has advantages over the use of hormones to induce superovulation *in vivo*. IVM does not have physiological requirements, such as a specific state of sexual maturity, and thus can be performed regardless of animal age [14, 15], and oocytes can be easily obtained from antral follicles of the ovaries [16]. High-quality IVM of guinea pig oocytes is essential for successful *in vitro* fertilization (IVF), embryo development, and cloning by nuclear transfer. However, compared with the relatively well-established IVM systems for other mammals, such as mice [17], cattle [18, 19], and sheep [20], the IVM of guinea pig oocytes has often been unsuccessful, with few studies reporting low and variable maturation efficiency [4]. This is likely because the reproductive characteristics of guinea pigs hinders the ability of their oocytes to undergo competent nuclear and cytoplasmic maturation to allow subsequent fertilization and culture of embryos *in vitro* [8, 21]. Gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) promote oocyte maturation in cattle, goats, and sheep and improve embryo quality and quantity after IVF [18, 20, 22, 23]. However, although IVM oocytes typically complete nuclear maturation, their state of cytoplasmic maturity is unknown, and their ability to exhibit embryonic development after fertilization is usually lower than that of oocytes matured *in vivo* [24, 25].

Guinea pigs mature sexually at 2 months of age, but they do not exhibit normal fertility and pregnancy until 4 months of age. With increased maternal age, the number of collected oocytes and their efficiencies of IVM and IVF decrease [26]. The effects of the first endogenous hormone wave on the stimulation and regulation of guinea pig oocytes and their progression through meiosis are unclear, and the effect of IVM on the meiosis of ovarian follicular oocytes across guinea pig sexual and physical maturation has not been reported. Thus, the maturation of guinea pig oocytes, including their progression through meiosis during IVM before IVF, must be studied in detail to generate fertilized embryos.

Leukemia inhibitory factor (LIF), which is expressed in ovaries, embryos, and endometrium, plays an important role in regulating follicular development [27], embryonic development and implantation, and trophoblast cell invasion and maintenance of pregnancy [28]. LIF induces the expansion of cumulus cells around human and mouse oocytes during their maturation and increases the two-cell and blastocyst ratios in mice, deer mice, and cattle after IVF [29, 30]. Cysteamine (Cys) reduces damage caused by the oxidative stress response of free oxygen radicals during cell metabolism by increasing levels of glutathione (GSH) in oocytes [20]. Cys supplementation in IVM medium improves the maturation efficiency of pig, cow, and goat oocytes and promotes formation of the male pronucleus and the embryonic developmental potential of IVF embryos [31, 32]. Y27632 is an ATP-competitive ROCK-I and ROCK-II inhibitor that influences cell proliferation, apoptosis, and microfilament assembly. ROCK regulates the location and migration of meiotic spindles by regulating the assembly of microfilaments in oocytes, thereby influencing their unequal division and polar body protrusion. Studies in mice, cattle, and pigs report that Y27632 inhibits oocyte maturation by preventing germinal vesicle (GV) breakdown via inhibition of ROCK expression [17, 33, 34]. Interestingly, one study reports that the beneficial effect of Y27632 on the vitrification and resuscitation of bovine oocytes may be due to reduced apoptosis and normalized function of the microtubule organizing center [35]. Similarly, we previously found that combined treatment with LIF, Cys, and Y27632 synergistically promotes the maturation of goat oocytes *in vitro* and subsequent development of embryos after IVF [20].

Here, we examined the isolated and combined effects of LIF, Cys, and Y27632 supplementation during IVM on the maturation rates of guinea pig oocytes. We also examined the effects of IVM duration and guinea pig age on the progression of oocyte meiosis as well as the effect of Y27632 on oocyte microfilament intensity during IVM.

Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

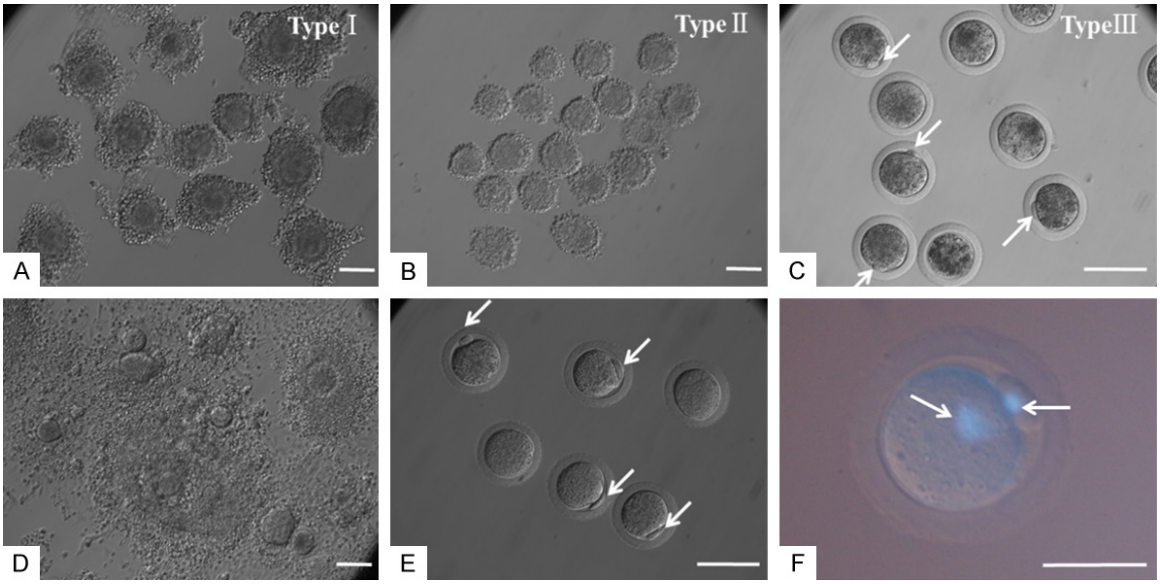

Figure 1. Types of guinea pig oocytes and their progression through meiosis during *in vitro* maturation. (A) Cumulus-oocyte complexes were collected from ovaries. Type I oocytes had four or more layers of cumulus cells. (B) Type II oocytes had one to three layers of cumulus cells. (C) Type III oocytes were denuded or had a few cumulus cells and exhibited the first polar body (arrows). (D) Type I oocytes were matured for 24 h *in vitro*, during which cumulus cells expanded and detached to the bottom of the culture dish. (E) After stripping off cumulus cells, oocytes began to show the first polar body (arrows). (F) Representative fluorescent microscopy image showing an oocyte with metaphase chromosome plates (arrows) in both oocyte and the polar body, indicating its progression to MII. Scale bars =100 μm in (A-E); 80 μm in (F).

Guinea pig use and maintenance

Guinea pig experimental protocols were approved by the Animal Care and Use Committee of Nanjing Normal University and complied with guidelines and standards from the U.S. National Institutes of Health. Two- or 4-month-old female guinea pigs with normal cycles were purchased from a certified animal facility. Females were maintained in an animal facility under a 12:12 light cycle at 22-23°C and 65% humidity. Guinea pigs were freely given commercial food pellets enriched with vitamin C and tap water containing 0.2 mg/ml vitamin C. Guinea pigs were monitored daily to detect vaginal opening, and vaginal smears were performed to identify the day of ovulation.

Oocyte collection and IVM

Guinea pig ovaries were collected and rinsed in pre-warmed (30°C) saline solution. Ovary tissue was sliced into small pieces to expose antral follicles to D-PBS-based oocyte aspirate plus (Renova Life Inc., College Park, MD) supplemented with 3 IU/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cumulus-oocyte complexes were recovered by puncturing the antral follicles by a 20 G needle under a stereomicroscope. Oocytes were classified into three types: type I oocytes had four or more layers of cumulus cells (**Figure 1A**); type II oocytes had one to three layers of cumulus cells (**Figure 1B**); and type III oocytes were denuded or had few cumulus cells scattered in the zona pellucida and exhibited the first polar body (**Figure 1C**).

Following selection, type I and II oocytes were washed three times in D-PBS-based oocyte aspirate plus solution and once in 10% fetal bovine serum (FBS; SH0070.03, Hyclone, Logan, UT) and 10% FBS M199 medium (basal medium) containing Earle's salts, L-glutamine, 2.2 g/l sodium bicarbonate, and 25 mM HEPES (Gibco, Grand Island, NY). Maturation medium (MAT medium) was basal M199 medium supplemented with 0.5 μg/ml ovine FSH (NIDDK), 5.0 μg/ml ovine LH (NIDDK), 1.0 μg/ml estradiol (E-8875), and antibiotics. Maturation droplets were pre-incubated under mineral oil and 5% CO_2 in humidified air at 37.5°C for at least 3 h before IVM. Treatments with different combinations of 100 μM Cys (M9768), 1000 IU/ml LIF (Invitrogen, Grand Island, NY), and 10 μM Y27632 (Renova Life Inc.) are described below.

Twenty to 25 oocytes were transferred into droplets of MAT medium (75 μl) and incubated at 37.5°C in 5% CO_2 in a humidified atmosphere for 24 h as previously described [20].

Examination of oocyte maturation status during IVM

After 24 h of IVM, oocytes (**Figure 1D**) were exposed to 3 mg/ml hyaluronidase in D-PBS for 3 min, after which cumulus cells were removed by pipetting and washing with 10% FBS M199 (**Figure 1E**). Oocyte nuclear maturation status was examined under a fluorescent microscope (Olympus BX53, Japan) after staining with 100 ng/ml 4',6'-diamidino-2-phenylindole, dihydrochloride (DAPI, BD5010, Bioworld, Nanjing, China) for 10 min (**Figure 1F**). Oocytes were characterized as being in GV, metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII) [36].

Spindle arrangement and microfilament intensity in oocytes during IVM

Oocytes were washed three times in D-PBS containing 0.1% polyvinyl alcohol (P8136) and fixed with 4% paraformaldehyde (16005) in D-PBS for 15 min. After washing in D-PBS for 10 min, fixed oocytes were permeabilized by incubation in 0.5% Triton-X100 (T8200, Solarbio, Shanghai, China) for 20 min and treated with 0.25% Tween 20 (9005-64-5, Sangon Biotech, Shanghai, China) in D-PBS under mineral oil (M8410) for 30 min. Oocytes were incubated with 2% FBS in D-PBS for 1 h at room temperature to block nonspecific binding sites and then incubated with a primary antibody for β-tubulin (1:200 dilution; BS1482M, Bioworld, Nanjing, China) in D-PBS supplemented with 2% FBS overnight at 4°C. For the control group, oocytes were incubated in blocking fluid overnight instead of primary antibody. Oocytes were washed in PBST three times for 5 min at room temperature and then incubated with secondary antibody goat anti-mouse IgG H&L (FITC; 1:1,000 dilution; ab6785 Abcam Trading (Shanghai) Company Ltd. Pudong, Shanghai, China) for 1 h at 37°C. Control group was treated without first antibody to β-tubulin, but with the secondary antibody to eliminate the background contamination (**Figure 2A1-D4**). Three replicates were performed.

For F-actin staining, oocytes were incubated with TRITC phalloidin (1:200 dilution; 40734-ES75, Yeasen, Shanghai, China) for 30 min at room temperature. Oocytes were then washed in PBST for 30 min and stained with 100 ng/ml DAPI for 10 min. After staining, oocytes were mounted on glass slides and examined with a fluorescence microscope (Olympus BX53, Japan). Control group was treated without TRITC phalloidin to eliminate the background contamination (**Figure 3A**). Three replicates were performed.

Experimental design

Effect of Cys, LIF, and Y27362 on oocyte maturation: To evaluate the effect of Cys, LIF, and Y27362 in isolation or combination on oocyte IVM, we performed a factorial experiment using different media: (1) M199 basal medium, (2) MAT (M199 basal medium containing FSH and LH), (3) MAT+Cys, (4) MAT+LIF, (5) MAT+Y27632, (6) MAT+Cys+LIF, or (7) MAT+Cys+LIF+Y27632. After 24 h of IVM, nuclear formation was evaluated by DAPI staining. Because type III oocytes underwent spontaneous maturation in ovarian follicles, only type I and II oocytes were used.

Effect of IVM duration on oocyte maturation: To determine the optimal IVM duration for the maturation of guinea pig oocytes, type I oocytes from 2-month-old guinea pigs were incubated in MAT+Cys+LIF medium for 24, 36, or 48 h, and nuclear formation was evaluated by DAPI staining.

Effect of guinea pig age on oocyte maturation: To examine the effect of guinea pig age on oocyte maturation, we incubated type I oocytes from 2- or 4-month-old guinea pigs in MAT+Cys+LIF medium for 24 h, and nuclear formation was evaluated by DAPI staining.

Effect of Y27632 on microfilaments during oocyte maturation: To explore the inhibitory effect of Y27632 on guinea pig oocyte maturation, type I oocytes from 2-month-old guinea pigs were matured in MAT or MAT+Y27632 medium for 24 h, and the reactive fluorescence intensity of F-actin was calculated.

Statistical analysis

Data on oocyte lysis, GV breakdown, MI, AI, TI and MII rates, and cleavage rate were analyzed using SPSS software (SPSS 20.0, Chicago, IL). Percentage data in each replicate were arcsine-transformed as mean ± standard error of the

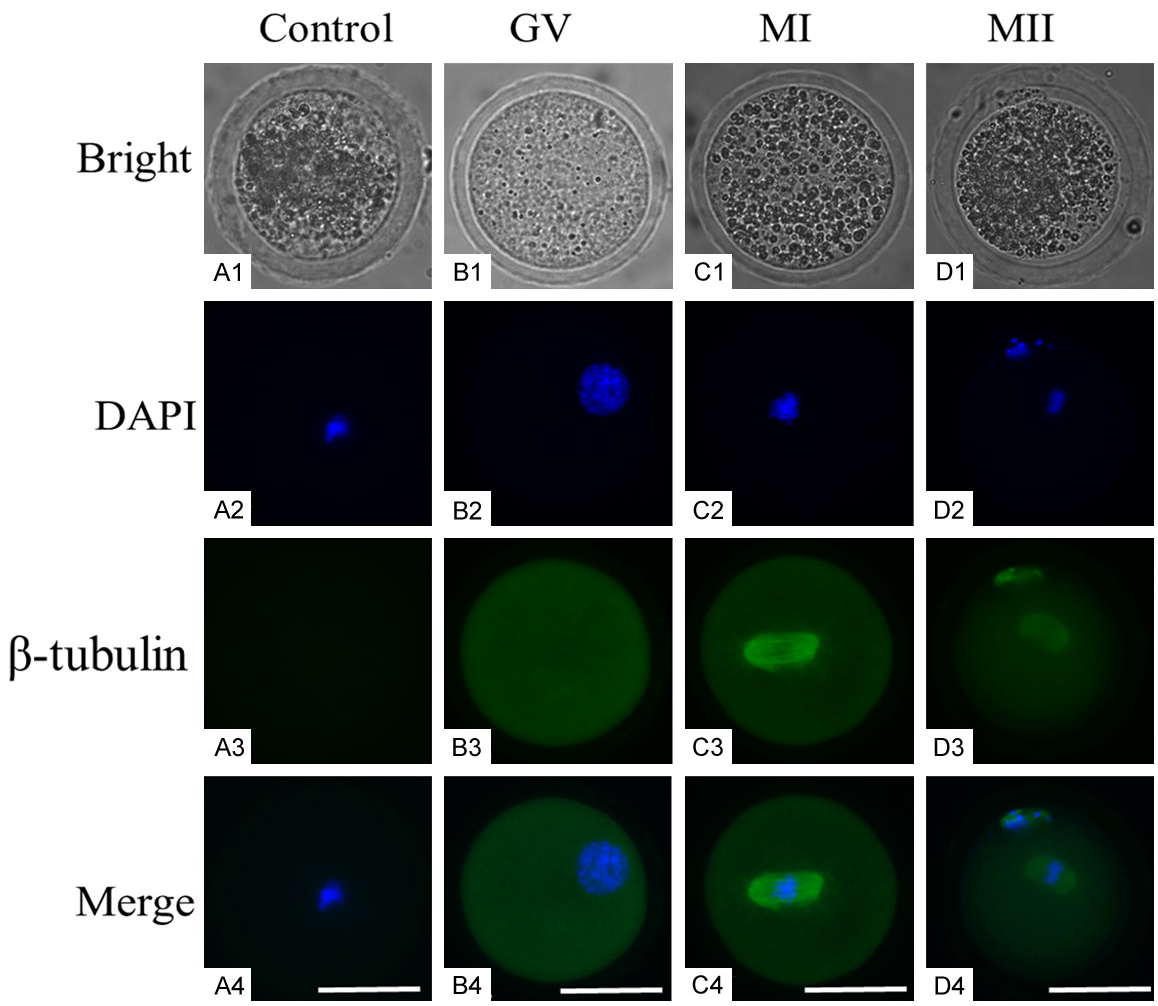

Figure 2. Characterization of guinea pig oocyte maturation during *in vitro* maturation (IVM). After 24 h of IVM, oocytes were stripped of cumulus cells and prepared for immunofluorescence microscopy. (A1-A4) Control oocytes were fixed and incubated only with secondary antibody before DAPI staining. (B1-B4) Germinal vesicle (GV) oocytes showed a large nuclear structure stained by DAPI but a lack of spindle apparatus labeled by β-tubulin. (C1-C4) Metaphase I (MI) oocytes showed a clear β-tubulin spindle across the chromosome plate in the middle of the oocyte. (D1-D4) In metaphase II (MII) oocytes, a first polar body was observed (D1), metaphase chromosomes were separated and displaced in both the polar body and the oocyte (D2), and β-tubulin spindles were arranged across the chromosome plates (D3 and D4). Control group was treated without first antibody to β-tubulin, but with the secondary antibody to eliminate the background contamination. Scale bars =50 μm.

mean (SEM) and subjected to one-way ANOVA. Pairwise comparisons were performed using Fisher's least significant difference tests. The average values of all F-actin fluorescence intensity measurements were used to compare final average intensities between control and treatment groups using ImageJ software. Statistical significance was set at $P<0.05$.

Results

From a total of 114 guinea pigs (86 and 28 animals at 2 and 4 month of age, respectively), we collected three types of oocytes from ovarian antral follicles: type I (n=1,650), type II (n=1,144), and type III (n=310). The average length, width, and volume of ovaries was 4.9 ± 0.1 mm, 3.4 ± 0.1 mm, and 30.5 ± 1.7 mm^3, respectively.

At the time of collection, oocytes were stripped of cumulus cells and stained with DAPI to examine their nuclear maturation. Most type I (95.2%) and II (92.1%) oocytes were in GV phase (**Figure 2B1-B4**) ($P>0.05$), with few in MI phase (**Table 1**). However, type III oocytes underwent spontaneous maturation in the follicles into different phases of meiosis, with

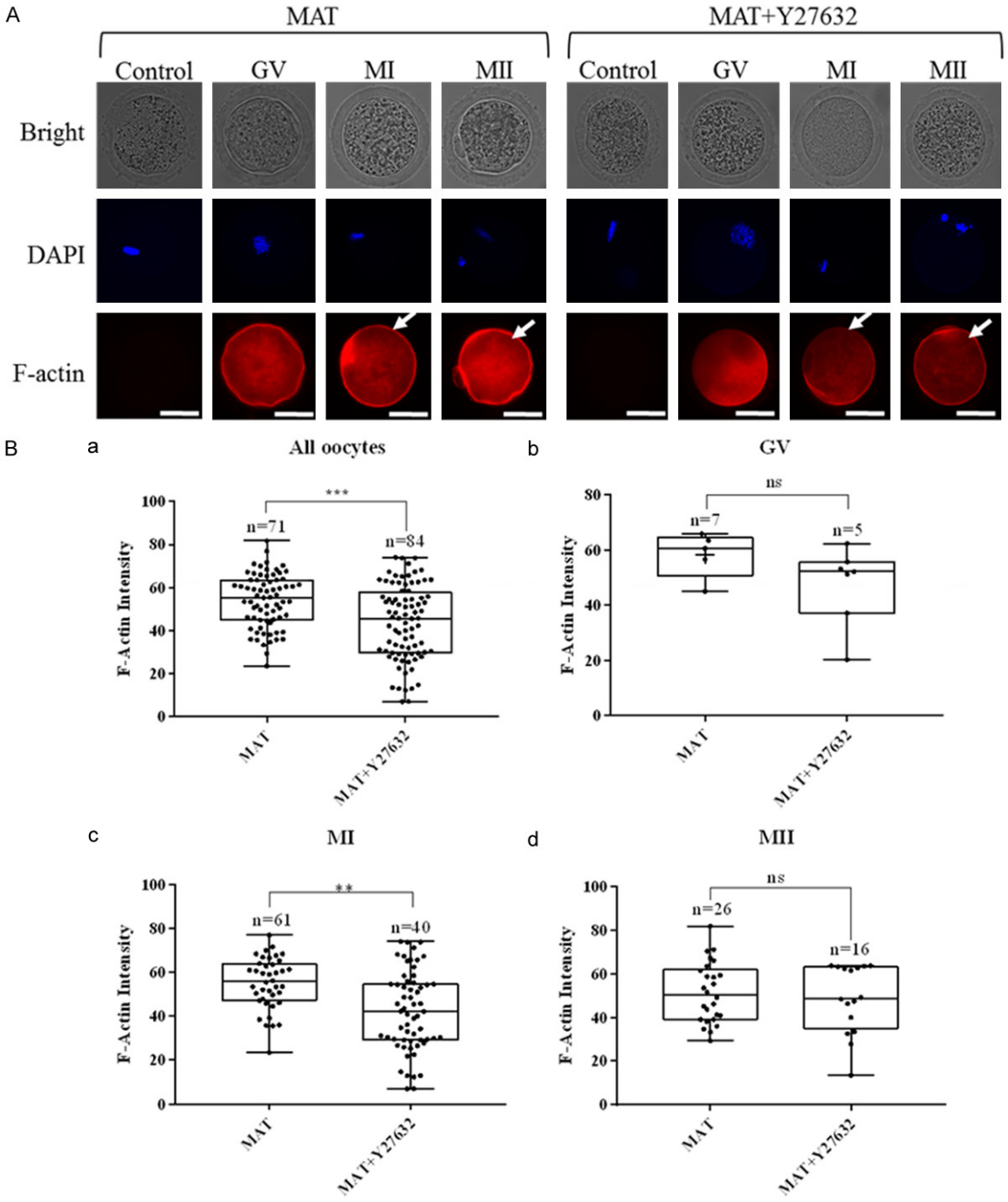

Figure 3. Effect of Y27632 on the presence of microfilaments in guinea pig oocytes during *in vitro* maturation. (A) Oocyte at metaphase I (MI) and metaphase II (MII) showed morphologically lower F-actin intensity (red) in the maturation medium (MAT)+Y27632 group than in the MAT group (arrows). Control group was treated without TRITC phalloidin to eliminate the background contamination. (B) F-actin intensity was significantly lower in the MAT+Y27632 group than in the MAT group regardless of oocyte meiotic phase (a; ***P<0.001). Germinal vesicle (GV; b) and MII (d) oocytes showed similar F-actin intensities between groups (ns, not significant; P>0.05), whereas MI oocytes showed a significantly lower F-actin intensity in the MAT+Y27632 group than in the MAT group (c; **P<0.01). Scale bars =50 μm.

many showing disturbed and scattered chromosomes (P<0.05). Also, more type III oocytes exhibited lysis (12.5%) than type I (2.4%) or II oocytes (2.3%) (P<0.05).

Table 1. Phases of meiosis progression of guinea pig oocytes during *in vitro* maturation

Oocyte Type	No. replicates	No. oocytes	Lysed (%)	GV (%)	MI (%)	AI (%)	TI (%)	MII (%)	Scattered chromosomes (%)
Type I	6	96	2.4 ± 2.4[a]	95.2 ± 2.4[a]	2.4 ± 1.5[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
Type II	6	93	2.3 ± 1.5[a]	92.1 ± 2.6[a]	5.6 ± 2.3[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
Type III	8	214	12.5 ± 1.9[b]	5.7 ± 2.2[b]	32.3 ± 2.7[b]	0.8 ± 0.6[a]	1.8 ± 0.7[b]	26.6 ± 5.3[b]	20.1 ± 3.8[b]

[a,b]Values with different superscript letters within columns are significantly different (P<0.05). AI, anaphase I; GV, germinal vesicle; MI, metaphase I; MII, metaphase II; TI, telophase I.

Table 2. Effect of Cys, LIF, and Y27632 on guinea pig oocytes during *in vitro* maturation

Type*	Treatment	No. replicates	No. oocytes	Lysed (%)	GV (%)	MI (%)	MII (%)
Type I	M199+10% FBS	4	75	20.5 ± 8.1[a]	14.7 ± 7.5[a]	67.8 ± 8.8[a]	17.5 ± 2.1[a]
	MAT	4	87	7.2 ± 3.8[a]	0.9 ± 0.9[a]	57.7 ± 4.3[a]	41.4 ± 3.7[b]
	MAT+Cys	4	71	7.8 ± 4.5[a]	1.4 ± 1.4[a]	59.2 ± 3.2[a]	39.5 ± 1.9[b]
	MAT+LIF	4	68	9.0 ± 4.2[a]	0.0[a]	59.2 ± 2.5[a]	40.9 ± 2.5[b]
	MAT+Y27632	4	64	4.9 ± 3.0[a]	3.9 ± 3.9[a]	68.5 ± 5.2[a]	27.7 ± 2.1[c]
	MAT+Cys+LIF	4	90	6.9 ± 3.1[a]	0.0[a]	38.3 ± 2.4[b]	61.8 ± 2.4[d]
	MAT+Cys+LIF+Y27632	4	84	2.3 ± 1.3[a]	3.8 ± 2.5[a]	66.5 ± 5.5[a]	29.7 ± 2.5[c]
Type II	M199+10% FBS	4	82	24.5 ± 11.6[a,b]	67.4 ± 13.3[a]	26.6 ± 10.5[a]	6.0 ± 3.0[a]
	MAT	4	75	13.1 ± 5.1[a,b]	39.9 ± 4.6[a]	40.5 ± 3.9[a]	19.7 ± 1.2[b,c]
	MAT+Cys	4	68	19.1 ± 4.5[a,b]	30.8 ± 8.2[a]	46.0 ± 8.6[a]	23.3 ± 1.8[b,d]
	MAT+LIF	4	82	17.0 ± 2.7[a,b]	37.5 ± 4.9[a]	39.3 ± 7.5[a]	23.2 ± 3.0[b,c,d]
	MAT+Y27632	4	70	11.4 ± 5.6[a,b]	36.1 ± 11.1[a]	48.8 ± 8.6[a]	15.2 ± 2.5[a,c]
	MAT+Cys+LIF	4	84	9.3 ± 4.7[a]	21.5 ± 8.5[b]	49.1 ± 8.2[a]	29.5 ± 2.7[d]
	MAT+Cys+LIF+Y27632	4	88	26.4 ± 4.4[b]	33.4 ± 13.4[a]	49.4 ± 10.6[a]	17.2 ± 2.9[b,c]

[a,b,c,d]Values with different superscript letters within columns are significantly different within type I and II classifications (P<0.05). *Maturation data were analyzed independently within type I and II classifications. Cys, cysteamine; GV, germinal vesicle; LIF, leukemia inhibitory factor; MAT, maturation medium; MI, metaphase I; MII, metaphase II.

Effect of Cys, LIF, and Y27362 on oocyte maturation

Among type I oocytes cultured in basal medium (M199+10% FBS), most stopped meiosis progression at MI phase (**Figure 2C1-C4**), with some at MII phase (17.5%) (**Figure 2D1-D4**; **Table 2**). Culture in MAT medium (containing LH and FSH) significantly increased the MII rate (41.4%) (P<0.05). When LIF, Cys, or Y27632 were supplemented into MAT medium in isolation, the MII rate was similar between MAT+Cys (39.5%) and MAT+LIF (40.9%) groups (P>0.05) but significantly lower in the MAT+Y27632 group (27.7%) (P<0.05). When Cys and LIF were supplemented in combination (MAT+Cys+LIF), the MII rate (61.8%) was significantly higher than that in all single factor groups (P<0.05). However, supplementation of Y27632 together with Cys and LIF (MAT+Cys+LIF+Y27632) significantly reduced the MII rate (29.7%) (P<0.05) and increased the MI rate (66.5%) (P<0.05).

Similar trends were observed among type II oocytes, although type II oocytes showed overall lower maturation rates than type I oocytes (P<0.05; **Table 2**). Higher MII rates were observed in the MAT+Cys, MAT+LIF, and MAT+Cys+LIF groups compared with the other groups. However, when Y27632 was supplemented in isolation (MAT+Y27632) or in combination (MAT+Cys+LIF+Y27632), MII phase rate significantly lower than that in the MAT+Cys+LIF group (P<0.05).

Effect of IVM duration on oocyte maturation

We examined the effect of IVM duration on type I oocytes cultured in MAT+Cys+LIF medium. Durations of 36 h and 48 h significantly increased the rate of oocyte lysis (13.7-23.6%) compared with 24 h (3.8%) (P<0.05; **Table 3**). Although there were no significant differences in MII rate among groups (43.7-54.5%) (P>0.05), the overall efficiency of maturation (calculated

Table 3. Effect of culture duration on guinea pig oocytes during *in vitro* maturation

Duration	No. replicates	No. oocytes	Lysed (%)	GV (%)	MI (%)	MII (%)	Efficiency (%)*
24 h	4	100	3.8 ± 2.8[a]	0.0a	45.5 ± 1.7[a]	54.5 ± 1.7[a]	52.5 ± 2.0[a]
36 h	4	117	13.7 ± 1.4[b]	0.0a	50.8 ± 2.3[a]	49.2 ± 2.3[a]	42.5 ± 2.5[b]
48 h	4	103	23.6 ± 4.5[b]	0.0a	56.3 ± 5.5[a]	43.7 ± 5.5[a]	32.8 ± 3.0[c]

[a,b,c]Values with different superscript letters within columns are significantly different (P<0.05). *means the over all efficiency of maturation (calculated as MII oocytes/all oocytes in culture). GV, germinal vesicle; MI, metaphase I; MII, metaphase II.

Table 4. Effect of animal age on guinea pig oocytes during *in vitro* maturation

Age	No. replicates	No. oocytes	Lysed (%)	GV (%)	MI (%)	MII (%)
2 months	4	87	6.5 ± 1.9[a]	0.0[a]	43.5 ± 3.6[a]	56.5 ± 3.6[a]
4 months	4	93	7.0 ± 4.1[a]	0.0[a]	55.3 ± 0.7[b]	44.8 ± 0.7[b]

[a,b]Values with different superscript letters within columns are significantly different (P<0.05). GV, germinal vesicle; MI, metaphase I; MII, metaphase II.

as MII oocytes/all oocytes in culture) was significantly higher for the 24 h group (52.5%) than the 36 h (42.5%) and 48 h (32.8%) groups (P<0.05).

Effect of guinea pig age on oocyte maturation

We next examined the effect of guinea pig age on oocyte IVM. The number of collected oocytes was similar between 2-month-old (50.2 ± 5.9; type I: 19.5 ± 1.9, type II: 22.2 ± 3.4) and 4-month-old (41.6 ± 5.1; type I: 15.6 ± 2.5, type II, 21.8 ± 2.6) guinea pigs (P>0.05; **Table 4**). After 24 h of IVM, the MII rate of type I oocytes (56.5%) was significantly higher in the 2-month-old group than in the 4-month-old group (44.8%) (P<0.05).

Effect of Y27632 on microfilaments during oocyte maturation

To explore the inhibitory effect of Y27632 of guinea pig oocyte maturation, we compared the distribution and intensity of F-actin in oocytes after 24 h of IVM in MAT and MAT+Y27632 medium. Morphologically, F-actin intensity appeared lower in the MAT+Y27632 group than in the MAT group (**Figure 3A**). Further analysis showed that the average F-actin intensity in all oocytes was significantly higher in the MAT group than in the MAT+27632 (P<0.001); **Figure 3Ba**). When oocytes at different meiotic phases were compared, F-actin intensity was similar between MAT and MAT+Y27632 groups among GV (P>0.05; **Figure 3Bb**) and MII (P>0.05; **Figure 3Bd**) oocytes. However, in MI oocytes, F-actin intensity was significantly lower in the MAT+Y27632 group than in the MAT group (P<0.01; **Figure 3Bc**).

Discussion

We found that the combined supplementation of Cys and LIF, but not Y27632, into IVM medium containing FSH and LH promoted the maturation of guinea pig oocytes *in vitro*. As many as 61.8% of type I oocytes achieved MII during 24 h of IVM in MAT+Cys+LIF medium. Supplementation with Y27632, however, inhibited the progression of oocytes through meiosis and their microfilament intensity.

The function of COCs is mediated by cell-to-cell communication involving gap junctions and signal transduction pathways [37]. Early studies show that oocyte growth and development depend upon the supply of nutrients from surrounding follicle cells [38]. In the present study, we collected and classified three types of oocytes from guinea pig ovarian follicles. Type I (i.e., four or more layers of cumulus cells) and type II (i.e., one to three layers of cumulus cells) oocytes were mostly at the transitional and tentative GV phase, whereas type III oocytes were either denuded or carried few scattered cumulus cells or had undergone spontaneous meiosis in the follicles to reach various phases of meiosis. This indicates that COC contact loosens when oocyte meiosis is restored in follicles, and guinea pig oocytes mature to MII before ovulation, similar to humans but different from dogs [39].

The competent maturation of oocytes and their quality are directly related to their capabilities for fertilization and subsequent embryonic development. Successful oocyte IVM is strictly regulated by many biological and environmental factors [20, 38], such as the density of cumulus cells around oocytes, the components of the maturation medium, and the presence of *in vitro* conditions that mimic those *in vivo*. Early studies show that cumulus cells play an important role in oocyte maturation [37]. Cumulus cells can maintain the stagnation of oocyte meiosis within follicles, particularly at the GV phase, are involved in the induction and regulation of oocyte meiosis, and provide nutrients for oocyte growth and development [40, 41]. We found that type I oocytes, which contained more layers of cumulus cells, had a higher maturation rate than type II oocytes, indicating that peripheral cumulus cells play an important role in oocyte maturation.

FSH and LH are key hormones for oocyte maturation *in vitro* [18, 42]. By binding to granulosa cell surface receptors, FSH and LH induce second messenger cAMP activity through G protein-coupled receptor signal transduction, which activates protein kinase A (PKA) and thereby increases the synthesis of estrogen and progesterone. Resultant cAMP is transported into oocytes from adjacent cumulus cells through gap junctions [43]. PKA regulates the activity of maturation promotion factor (MPF), possibly through dephosphorylation of CDK1 by phosphatase CDC25 [44]. As a result, dephosphorylation of CDK1 activates MPF and promotes oocyte GV breakdown [43]. By contrast, a high level of cAMP leads to phosphorylation of CDK1, resulting in MPF inactivation and arrest of oocytes at the GV phase [43]. At the same time, the gap junction protein Cx43 becomes phosphorylated, and gap junctions close [45], which reduces the level of cAMP in oocytes. As a result, MPF activity is restored, inducing resumption of meiosis [43, 44]. Although the effects of FSH and LH on cAMP function during oocyte maturation may seem puzzling, they could be explained by threshold levels of cAMP in regulating oocyte maturation; that is, lower cAMP levels may initiate the process of maturation, but higher cAMP levels may inhibit the MPF activity that blocks oocyte meiosis. This possibility warrants further extensive study. In the present study, FSH and LH promoted guinea pig oocyte maturation during IVM, similar to that observed for bovine oocytes [18], with a greater effect in type I oocytes than in type II oocytes.

LIF is a multi-effector molecule that plays an important role in regulating follicular development [27], embryonic development and implantation [46], and trophoblast cell invasion and maintenance of pregnancy [28]. LIF activates downstream JAK/STAT3 and Ras-MAPK signaling pathways by binding to its receptors (LIFR and gp130). Early studies show that MAT medium supplemented with LIF promotes oocyte maturation and enhances their developmental capability through Ras-MAPK and/or JAK/STAT3 signaling pathways, but the specific underlying mechanisms remain unclear. In the present study, we found no effect of LIF on the MII rate of guinea pig oocytes during IVM. Similarly, adding LIF to MAT medium slightly improved the ability of goat oocytes to reach MII but did not improve fertilization or blastocyst rates [20]. In our future study, we will examine whether LIF transduces the JAK-STAT cascade to regulate the expression of important genes such as OCT4 and Sox2, as OCT4 is widely distributed in rabbit MII oocyte cytoplasm [47]. In addition, it remains to be determined whether the LIF-JAK-ERK-Fos-Jun pathway influences the expression of c-Mos [48].

Cys improves pronuclear formation, cleavage, and further embryonic development [31, 32, 49, 50]. Cys has reported to reduce oocyte damage caused by oxidative stress during cell metabolism via increasing the level of GSH [20]. Oxidative stress, which can induce oocyte and early embryo apoptosis, is mediated by reactive oxygen species, which are involved in a variety of physiological reproductive functions including oocyte maturation, hormone production, and luteal function. GSH accumulation in oocytes is necessary for fertilization and sperm nuclear de-condensation [51]. In the present study, supplementation of Cys alone in MAT medium did not increase the MII rate of oocytes, perhaps because Cys requires the assistance of other molecules to promote oocyte maturation.

We found that combined Cys and LIF supplementation promoted the nuclear progression of guinea pig oocytes to MII. This synergistic effect of Cys and LIF suggests that the antioxidant Cys reduced the amount of oxidative stress,

which further synergized and amplified the function of LIF-induced signal transduction. Due to the complexity of oocyte maturation in guinea pigs, the interactions between Cys and LIF that influence molecular networks and affect cellular events in oocytes are not yet known, but it may involve interactions between granulosa cells and oocytes, and the participation of Cys in the LIF signaling pathway during maturation. We propose that Cys increases oocyte GSH concentration through COC gap junctions or direct transportation, as it was found that this suitable intracellular antioxidant condition improves maturation-related gene expression through LIF signal transduction during oocyte maturation [20, 52]. Our finding of a synergistic effect of Cys and LIF on guinea pig oocyte maturation lays a foundation for further studies of the molecular mechanisms of oocyte maturation.

Importantly, however, supplementation of Y27632 into MAT medium inhibited the maturation of type I and II oocytes by reducing microfilament intensity and blocking progression from MI to MII. ROCK, an effector of small GTPase Rho, belongs to the AGC family of serine/threonine kinases and consists of ROCK-I and ROCK-II. ROCK indirectly coordinates a variety of cellular processes, such as cell morphology, cytokinesis, and movement, and plays an important role in the formation of F-actin fibers and F-actin dynamics [53]. Major downstream substrates of the ROCK signaling pathway include myosin light chain and LIM domain kinase (LIMK). By regulating MLC2 phosphorylation and peripheral F-actin shrinkage, ROCK-I participates in the contraction of actomyosin in the cytoskeleton, whereas ROCK-II stabilizes the F-actin cytoskeleton by regulating cofilin phosphorylation through LIMK [54]. Through both effects, ROCK regulates the asymmetric division of oocytes, resulting in spindle migration to the edge of oocytes and formation of the first polar body [33]. The ROCK pathway is also closely related to the induction of apoptosis. Elevated caspase activity increases its mediated activation of ROCK-I cleavage, which accelerates the apoptosis process [55, 56]. Y27632 is a type of ATP-competitive ROCK-I and ROCK-II inhibitor that plays an important role in cell proliferation, apoptosis, and microfilament assembly. Y27632 promotes the proliferation of human embryonic stem cells, reduces separation-induced apoptosis, and maintains the pluripotency of stem cells [57]. However, the discrepant effects of Y27632 on oocyte maturation described in the literature indicate the presence of distinct species differences. Y27632 improves the developmental ability of vitrified bovine oocytes post-IVF by reducing oocyte apoptosis [35], and Cys, LIF, and Y27632 have a synergistic beneficial effect on goat oocyte maturation and embryo development [20]. By contrast, studies in mice, cattle, and pigs report that Y27632 inhibits oocyte maturation by reducing ROCK activity [17, 33, 34]. In the present study, Y27632 inhibited oocyte maturation and microfilament intensity during IVM. We speculate that Y27632 inhibits the assembly of microfilaments during the maturation of guinea pig oocytes through the LIMK-cofilin pathway. As a result, reduced levels of F-actin prevent progression from MI to MII and the protrusion of the first polar body. The inhibitory effect of Y27632 on meiotic chromosome separation in guinea pigs and its underlying molecular mechanisms will be studied in detail in future studies, such as those testing whether overexpression of cofilin and/or F-actin via mRNA expression vectors can resume the successful completion of oocyte meiosis in the presence of Y27632.

To determine the optimal conditions for IVF, we examined the effect of culture duration on oocyte maturation efficiency and found 24 h of IVM allowed the highest maturation efficiency. With longer periods of IVM, however, oocyte lysis increased and maturation decreased. This indicates that 24 h of IVM is sufficient to allow competent oocytes to complete meiosis and these oocytes might undergo apoptosis over longer IVM durations, reducing overall maturation efficiency. It is interesting to note that the MII rate was higher in oocytes from 2-month-old guinea pigs than those from 4-month-old animals. Guinea pigs reach sex maturity at 2 months of age and mature physically at 4 months of age, at which point they exhibit normal fertility and competent pregnancies. As the first endogenous wave of hormones impacts oocyte quality and complicates superovulation in mice, inducing superovulation in pre-puberty mice provides a larger number and higher quality of oocytes or zygotes [58]. In the present study, oocytes from younger guinea pigs exhibited better IVM, perhaps because 4-month-old animals had been impacted by endogenous hormone waves to their follicles. It is not clear,

however, whether the guinea pigs tested at 2 months of age were in puberty or pre-puberty or if they had been impacted by endogenous hormones. In the future, we will compare IVM efficiency before, during, and after puberty (with or without the first wave of endogenous hormone shock) to obtain information that can be used to more efficiently retrieve guinea pig oocytes for successful IVM and competent IVF.

In conclusion, this study is the first to report that combined supplementation of Cys and LIF, but not Y27632, into maturation medium containing FSH and LH improves the maturation efficiency of guinea pig oocytes, allowing them to successfully mature to MII. We also found that guinea pig oocyte maturation is improved when oocytes are collected from 2-month-old animals and cultured for 24 h. Furthermore, Y27632 blocked the progression of oocytes from MI to MII, possibly by reducing the presence of microfilaments. Our study provides an important scientific basis for further *in vitro* fertilization and embryo transfer, somatic cell nuclear transfer (i.e., cloning), and gene editing in guinea pigs by establishing an animal model of human reproductive biology and related human diseases.

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Disclosure of conflict of interest

None.

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References

[1] Grégoire A, Allard A, Huamán E, León S, Silva RM, Buff S, Berard M and Joly T. Control of the estrous cycle in guinea-pig (Cavia porcellus). Theriogenology 2012; 78: 842-847.

[2] Joshi HS, Watson DJ and Labhsetwar AP. Ovarian secretion of oestradiol, oestrone, 20-dihydroprogesterone and progesterone during the oestrous cycle of the guinea-pig. J Reprod Fertil 1973; 35: 177-181.

[3] van Kan CM, de Vries JI, Lüchinger AB, Mulder EJ and Taverne MA. Ontogeny of fetal movements in the guinea pig. Physiol Behav 2009; 98: 338-344.

[4] Suzuki O, Koura M, Noguchi Y, Takano K, Yamamoto Y and Matsuda J. Optimization of superovulation induction by human menopausal gonadotropin in guinea pigs based on follicular waves and FSH-receptor homologies. Mol Reprod Dev 2003; 64: 219-225.

[5] Bland KP. Biphasic follicular growth in the guinea-pig oestrous cycle. J Reprod Fertil 1980; 60: 73-76.

[6] Hutz RJ, Bejvan SM, Durning M and Dierschke DJ. Changes in follicular populations, in serum estrogen and progesterone, and in ovarian steroid secretion in vitro during the guinea pig estrous cycle. Biol Reprod 1990; 42: 266-72.

[7] Feinberg RF, Kliman HJ and Wang CL. Transforming growth factor-beta stimulates trophoblast oncofetal fibronectin synthesis in vitro: implications for trophoblast implantation in vivo. J Clin Endocrinol Metab 1994; 78: 1241-1248.

[8] Suzuki O, Ogura A, Asano T, Noguchi Y, Yamamoto Y and Oike M. Development of preimplantation guinea-pig embryos in serum-free media. Reprod Fertil Dev 1993; 5: 425-432.

[9] Donovan BT and Lockhart AN. The hormonal induction of ovulation or of luteinization in the intact of hypophysectomized guinea-pig. J Endocrinol 1974; 60: 285-92.

[10] Peddie MJ. Follicular development in the adult guinea-pig and responses to human chorionic gonadotrophin. J Endocrinol 1980; 85: 9-16.

[11] Reed M and Hounslow WF. Induction of ovulation in the guinea-pig. J Endocrinol 1971; 49: 203-11.

[12] Shi F, Ozawa M, Komura H, Watanabe G, Tsonis CG, Suzuki AK and Taya K. Induction of superovulation by inhibin vaccine in cyclic guinea-pigs. J Reprod Fertil 2000; 118: 1-7.

[13] Li JR, Wang W and Shi FX. Induction of follicular luteinization by equine chorionic gonadotropin in cyclic guinea pigs. J Zhejiang Univ Sci B 2015; 16: 980-990.

[14] Khatun M, Bhuiyan MM, Ahmed JU, Haque A, Rahman MB and Shamsuddin M. In vitro maturation and fertilization of prepubertal and pu-

bertal black Bengal goat oocytes. J Vet Sci 2011; 12: 75-82.
[15] Jimenezmacedo AR, Izquierdo D, Urdaneta A, Anguita B and Paramio MT. Effect of roscovitine on nuclear maturation, MPF and MAP kinase activity and embryo development of prepubertal goat oocytes. Theriogenology 2006; 65: 1769-1782.
[16] Souza-Fabjan JM, Locatelli Y, Duffard N, Corbin E, Touzé JL, Perreau C, Beckers JF, Freitas VJ and Mermillod P. In vitro embryo production in goats: slaughterhouse and laparoscopic ovum pick up-derived oocytes have different kinetics and requirements regarding maturation media. Theriogenology 2014; 81: 1021-1031.
[17] Duan X, Liu J, Dai XX, Liu HL, Cui XS, Kim NH, Wang ZB, Wang Q and Sun SC. Rho-GTPase effector ROCK phosphorylates cofilin in actin-meditated cytokinesis during mouse oocyte meiosis. Biol Reprod 2014; 90: 37.
[18] An LY, Chaubal SA, Liu Y, Chen Y, Nedambale TL, Xu J, Xue F, Moreno JF, Tao S, Presicce GA and DU F. Significant heparin effect on bovine embryo development during sexed in vitro fertilization. J Reprod Dev 2017; 63: 175-183.
[19] Pellegrino CA, Morotti F, Untura RM, Pontes JH, Pellegrino MF, Campolina JP, Seneda MM, Barbosa FA and Henry M. Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of in vitro-produced embryos in cattle. Theriogenology 2016; 86: 888-893.
[20] An L, Liu J, Du Y, Liu Z, Zhang F, Liu Y, Zhu X, Ling P, Chang S, Hu Y, Li Y, Xu B, Yang L, Xue F, Presicce GA and Du F. Synergistic effect of cysteamine, leukemia inhibitory factor, and Y27632 on goat oocyte maturation and embryo development in vitro. Theriogenology 2018; 108: 56-62.
[21] Yanagimachi R. Maturation and fertilization in vitro of guinea-pig ovarian oocytes. J Reprod Fertil 1974; 38: 485-488.
[22] Dinopoulou V, Drakakis P, Kefala S, Kiapekou E, Bletsa R, Anagnostou E, Kallianidis K and Loutradis D. Effect of recombinant-LH and hCG in the absence of FSH on in vitro maturation (IVM) fertilization and early embryonic development of mouse germinal vesicle (GV)-stage oocytes. Reprod Biol 2016; 16: 138-146.
[23] Zhang Y, Duan X, Cao R, Liu HL, Cui XS, Kim NH, Rui R and Sun SC. Small GTPase RhoA regulates cytoskeleton dynamics during porcine oocyte maturation and early embryo development. Cell Cycle 2014; 13: 3390-3403.
[24] Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod Fertil Dev 1996; 8: 485-489.
[25] van de Leemput EE, Vos PL, Zeinstra EC, Bevers MM, van der Weijden GC and Dieleman SJ. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. Theriogenology 1999; 52: 335-349.
[26] Wu J, Zhang L and Wang X. Maturation and apoptosis of human oocytes in vitro are age-related. Fertil Steril 2000; 74: 1137-1141.
[27] Nilsson EE, Kezele P and Skinner MK. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. Mol Cell Endocrinol 2002; 188: 65-73.
[28] Fukuda MN and Sugihara K. Signal transduction in human embryo implantation. Cell Cycle 2007; 6: 1153-1156.
[29] De Matos DG, Miller K, Scott R, Tran CA, Kagan D, Nataraja SG, Clark A and Palmer S. Leukemia inhibitory factor induces cumulus expansion in immature human and mouse oocytes and improves mouse two-cell rate and delivery rates when it is present during mouse in vitro oocyte maturation. Fertil Steril 2008; 90: 2367-2375.
[30] Choi JK and He X. Improved oocyte isolation and embryonic development of outbred deer mice. Sci Rep 2015; 5: 12232.
[31] Grupen CG, Nagashima H and Nottle MB. Cysteamine enhances in vitro development of porcine oocytes matured and fertilized in vitro. Biol Reprod 1995; 53: 173-8.
[32] Urdaneta A, Jiménez-Macedo AR, Izquierdo D and Paramio MT. Supplementation with cysteamine during maturation and embryo culture on embryo development of prepubertal goat oocytes selected by the brilliant cresyl blue test. Zygote 2003; 11: 347-54.
[33] Lee SR, Xu YN, Jo YJ, Namgoong S and Kim NH. The Rho-GTPase effector ROCK regulates meiotic maturation of the bovine oocyte via myosin light chain phosphorylation and cofilin phosphorylation. Mol Reprod Dev 2016; 82: 849-858.
[34] Zhang Y, Duan X, Xiong B, Cui XS, Kim NH, Rui R and Sun SC. ROCK inhibitor Y-27632 prevents porcine oocyte maturation. Theriogenology 2014; 82: 49-56.
[35] Hwang IS, Hara H, Chung HJ, Hirabayashi M and Hochi S. Rescue of vitrified-warmed bovine oocytes with rho-associated coiled-coil kinase inhibitor. Biol Reprod 2013; 89: 26.
[36] An L, Chang S, Hu Y, Li Y, Xu B, Zhang F, Yang L, Presicce GA and Du F. Efficient cryopreservation of mouse embryos by modified droplet vitrification (MDV). Cryobiology 2015; 71: 70-76.
[37] Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL and Schoolcraft WB. Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. Fertil Steril 2015; 103: 303-316.

[38] Paramio MT and Izquierdo D. Recent advances in in vitro embryo production in small ruminants. Theriogenology 2016; 86: 152-159.
[39] Nagashima JB, Sylvester SR, Nelson JL, Cheong SH, Mukai C, Lambo C, Flanders JA, Meyers-Wallen VN, Songsasen N and Travis AJ. Live births from domestic dog (Canis familiaris) embryos produced by in vitro fertilization. PLoS One 2015; 10: e0143930.
[40] Appeltant R, Somfai T, Nakai M, Bodó S, Maes D, Kikuchi K and Soom AV. Interactions between oocytes and cumulus cells during invitro maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. Theriogenology 2015; 83: 567-576.
[41] Geshi M, Takenouchi N, Yamauchi N and Nagai T. Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. Biol Reprod 2000; 63: 1730-1734.
[42] Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, Moreno JF, Dinnyés A, Ji W, Tian XC, Yang X and Du F. Developmental potential of vitrified holstein cattle embryos fertilized in vitro with sex-sorted sperm. J Dairy Sci 2006; 89: 2510-2518.
[43] Zhang M, Ouyang H and Xia G. The signal pathway of gonadotrophins-induced mammalian oocyte meiotic resumption. Mol Hum Reprod 2009; 15: 399-409.
[44] Liu L, Kong N, Xia G and Zhang M. Molecular control of oocyte meiotic arrest and resumption. Reprod Fertil Dev 2013; 25: 463-471.
[45] Norris RP, Marina F, Mehlmann LM, Cowan AE, Simon AM, Paul DL, Lampe PD and Jaffe LA. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development 2008; 135: 3229-38.
[46] Aghajanova L. Leukemia inhibitory factor and human embryo implantation. Ann N Y Acad Sci 2010; 1034: 176-183.
[47] Chen CH, Chang WF, Liu CC, Su HY, Shyue SK, Cheng WT, Chen YE, Wu SC, Du F, Sung LY and Xu J. Spatial and temporal distribution of Oct-4 and acetylated H4K5 in rabbit embryos. Reprod Biomed Online 2012; 24: 433-442.
[48] Gilbert SF and Barresi MJF. Developmental biology, 11th Edition 2016. Am J Med Genet A 2017; 173: 1430-1430.
[49] Merton JS, Knijn HM, Flapper H, Dotinga F, Roelen BA, Vos PL and Mullaart E. Cysteamine supplementation during in vitro maturation of slaughterhouse- and opu-derived bovine oocytes improves embryonic development without affecting cryotolerance, pregnancy rate, and calf characteristics. Theriogenology 2013; 80: 365-371.
[50] Sovernigo TC, Adona PR, Monzani PS, Guemra S, Barros F, Lopes FG and Leal C. Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production. Reprod Domest Anim 2017; 52: 561-569.
[51] Zhou P, Wu YG, Li Q, Lan GC, Wang G, Gao D and Tan JH. The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes. Reproduction 2008; 135: 605-11.
[52] de Souza-Fabjan JM, Panneau B, Duffard N, Locatelli Y, de Figueiredo JR, Freitas VJ and Mermillod P. Invitro production of small ruminant embryos: late improvements and further research. Theriogenology 2014; 81: 1149-1162.
[53] Sun QY and Schatten H. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. Reproduction 2006; 131: 193-205.
[54] Shi J, Wu X, Surma M, Vemula S, Zhang L, Yang Y, Kapur R and Wei L. Distinct roles for ROCK1 and ROCK2 in theregulation of cell detachment. Cell Death Dis 2013; 4: e483.
[55] Chang J, Xie M, Shah VR, Schneider MD, Entman ML, Wei L and Schwartz RJ. Activation of Rho-associated coiled-coil protein kinase 1 (ROCK-1) by caspase-3 cleavage plays an essential role in cardiac myocyte apoptosis. Proc Natl Acad Sci U S A 2006; 103: 14495-500.
[56] Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A and Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol 2001; 3: 339-345.
[57] Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K and Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 2007; 25: 681-6.
[58] Wu B, Shi Y, Gong X, Yu L, Chen Q, Wang J and Sun Z. Evaluation of follicular synchronization caused by estrogen administration and its reproductive outcome. PLoS One 2015; 10: e0127595.