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 immu...
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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 hinder 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).
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**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% CO₂ 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% CO2 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
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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 \( 4.9 \pm 0.1 \text{ mm} \), \( 3.4 \pm 0.1 \text{ mm} \), and \( 30.5 \pm 1.7 \text{ mm}^3 \), 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 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.
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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

<table>
<thead>
<tr>
<th>Oocyte Type</th>
<th>No. replicates</th>
<th>No. oocytes</th>
<th>Lysed (%)</th>
<th>GV (%)</th>
<th>MI (%)</th>
<th>Al (%)</th>
<th>Ti (%)</th>
<th>MII (%)</th>
<th>Scattered chromosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>6</td>
<td>96</td>
<td>2.4 ± 2.4*</td>
<td>95.2 ± 2.4*</td>
<td>2.4 ± 1.5*</td>
<td>0.0*</td>
<td>0.0*</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Type II</td>
<td>6</td>
<td>93</td>
<td>2.3 ± 1.5*</td>
<td>92.1 ± 2.6*</td>
<td>5.6 ± 2.3*</td>
<td>0.0*</td>
<td>0.0*</td>
<td>0.0*</td>
<td>0.0*</td>
</tr>
<tr>
<td>Type III</td>
<td>8</td>
<td>214</td>
<td>12.5 ± 1.9*</td>
<td>5.7 ± 2.2*</td>
<td>32.3 ± 2.7*</td>
<td>0.8 ± 0.6*</td>
<td>1.8 ± 0.7*</td>
<td>26.6 ± 5.3*</td>
<td>20.1 ± 3.8*</td>
</tr>
</tbody>
</table>

*Values with different superscript letters within columns are significantly different (P<0.05; 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

<table>
<thead>
<tr>
<th>Type*</th>
<th>Treatment</th>
<th>No. replicates</th>
<th>No. oocytes</th>
<th>Lysed (%)</th>
<th>GV (%)</th>
<th>MI (%)</th>
<th>MII (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>M199+10% FBS</td>
<td>4</td>
<td>75</td>
<td>20.5 ± 8.1*</td>
<td>14.7 ± 7.5*</td>
<td>67.8 ± 8.8*</td>
<td>17.5 ± 2.1*</td>
</tr>
<tr>
<td></td>
<td>MAT</td>
<td>4</td>
<td>87</td>
<td>7.2 ± 3.8*</td>
<td>0.9 ± 0.9*</td>
<td>57.7 ± 4.3*</td>
<td>41.4 ± 3.7*</td>
</tr>
<tr>
<td></td>
<td>MAT+Cys</td>
<td>4</td>
<td>71</td>
<td>7.8 ± 4.5*</td>
<td>1.4 ± 1.4*</td>
<td>59.2 ± 3.2*</td>
<td>39.5 ± 1.9*</td>
</tr>
<tr>
<td></td>
<td>MAT+LIF</td>
<td>4</td>
<td>68</td>
<td>9.0 ± 4.2*</td>
<td>0.0*</td>
<td>59.2 ± 2.5*</td>
<td>40.9 ± 2.5*</td>
</tr>
<tr>
<td></td>
<td>MAT+Y27632</td>
<td>4</td>
<td>64</td>
<td>4.9 ± 3.0*</td>
<td>3.9 ± 3.9*</td>
<td>68.5 ± 5.2*</td>
<td>27.7 ± 2.1*</td>
</tr>
<tr>
<td></td>
<td>MAT+Cys+LIF</td>
<td>4</td>
<td>90</td>
<td>6.9 ± 3.1*</td>
<td>0.0*</td>
<td>38.3 ± 2.4*</td>
<td>61.8 ± 2.4*</td>
</tr>
<tr>
<td></td>
<td>MAT+Cys+LIF+Y27632</td>
<td>4</td>
<td>84</td>
<td>2.3 ± 1.3*</td>
<td>3.8 ± 2.5*</td>
<td>66.5 ± 5.5*</td>
<td>29.7 ± 2.5*</td>
</tr>
</tbody>
</table>

*Type II | M199+10% FBS | 4              | 82          | 24.5 ± 11.6*| 67.4 ± 13.3*| 26.6 ± 10.5*| 6.0 ± 3.0* |
|       | MAT       | 4              | 75          | 13.1 ± 5.1*| 39.9 ± 4.6*| 40.5 ± 3.9*| 19.7 ± 1.2* |
|       | MAT+Cys   | 4              | 68          | 19.1 ± 4.5*| 30.8 ± 8.2*| 46.0 ± 8.6*| 23.3 ± 1.8* |
|       | MAT+LIF   | 4              | 82          | 17.0 ± 2.7*| 37.5 ± 4.9*| 39.3 ± 7.5*| 23.2 ± 3.0* |
|       | MAT+Y27632| 4              | 70          | 11.4 ± 5.6*| 36.1 ± 11.1*| 48.8 ± 8.6*| 15.2 ± 2.5* |
|       | MAT+Cys+LIF| 4              | 84          | 9.3 ± 4.7* | 21.5 ± 8.5*| 49.1 ± 8.2*| 29.5 ± 2.7* |
|       | MAT+Cys+LIF+Y27632| 4              | 88          | 26.4 ± 4.4*| 33.4 ± 13.4*| 49.4 ± 10.6*| 17.2 ± 2.9* |

*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 Y27632 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%). Although there were no significant differences in MI rate among groups (43.7-54.5%) (P>0.05), lower maturation rates than type I oocytes 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...
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+Y27632 (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].
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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, cytoskeleton, 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 IVF, 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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