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—Full Paper—

Bovine Oocytes and Early Embryos Express mRNA Encoding Glycerol Kinase but Addition of Glycerol to the Culture Media Interferes with Oocyte Maturation

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Abstract. Glycerol plays multi-functional roles in cellular physiology. Other than forming the backbone molecule for glycerophospholipid and triglyceride (TG), glycerol acts as an energy substrate for glycolysis. Spermatozoa are known to utilize glycerol for energy production, but there are no reports of this in oocytes. In this study, the value of glycerol as an energy substrate for bovine oocyte maturation (Exp. 1) and the gene expression of glycerol kinase (GK), an enzyme crucial for cellular glycerol utilization, in bovine oocytes and early embryos (Exp. 2) were examined. In Exp. 1, *in vitro* maturation (IVM) was conducted using synthetic oviduct fluid supplemented with/without glucose (1.5 mM) and/or glycerol (1.0 mM), and maturation rate, degree of cumulus expansion, glucose consumption and lactate production by cumulus-oocyte complexes (COC) were examined. In Exp. 2, to examine the developmental expression of GK mRNA, cumulus cells, oocytes and embryos at the 2-, 8- and 16-cell, morula, expanded blastocyst and hatched blastocyst stages were obtained in separate experiments, and the expression of GK mRNA was quantified using a real-time PCR. Glycerol did not support oocyte maturation or cumulus expansion. Addition of glycerol to glucose-supplemented media significantly decreased the maturation rate. Expression of GK mRNA was very low in cumulus cells, whereas an appreciable level of the transcript was observed in the oocytes. GK mRNA was detected in embryos at all the stages examined, and its expression significantly increased at the morula stage. These results indicate that glycerol, at least at the present concentration, is not beneficial as a constituent of the medium for bovine oocyte maturation. However, the appreciable levels of GK mRNA found in the oocyte and embryo imply a physiological role for glycerol in bovine oocyte maturation and embryo development.

Key words: Bovine, Embryo, Glycerol, Glycerol kinase (GK), Oocyte maturation

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To understand the nutritional requirements of oocytes and embryos, studies on metabolism of energy substrates such as glucose, pyruvate, lactate and amino acid have been performed on the bovine oocytes and early embryos [1, 2]. Thanks to these studies, it has been clarified that the bovine oocyte and embryo exhibit dynamic changes in energy metabolism during the maturation and preimplantation period [1, 2]. During maturation, the oocyte cannot utilize glucose [3], and energy is mainly produced by oxidation of pyruvate supplied by the surrounding cumulus cells through glycolysis [4, 5]. Likewise, the early cleavage-stage embryo (up to 8–16 cells) has low glucose metabolic activity and mainly relies on oxidative phosphorylation for generation of over 90% of ATP [6]. At this stage, glucose metabolism occurs effectively via the pentose phosphate pathway to generate NADPH and ribose sugars [7]. At the 8- to 16-cell stages, there is a switch from pyruvate-based metabolism to glucose-based metabolism, which coincides with the maternal-zygotic transition (MZT). Subsequently, glucose is metabolized almost exclusively via the glycolytic pathway [7].

Glycerol, a polyalcohol, is essential for providing the backbone of glycerophospholipid and triglyceride [8, 9]. Glycerol is also utilized as a substrate for glycolysis [9]. Experiments on glycerol metabolism by ram and bull spermatozoa indicate that spermatozoa can utilize glycerol as an energy source through glycolysis [10–12]. However, there are no reports verifying the metabolism of glycerol in oocytes or early embryos despite the fact that it has been widely used as a cryoprotectant in cryopreservation of embryos.

The first step of glycerol metabolism is catalyzed by the glycerol kinase (GK) that phosphorylates glycerol to glycerol-3-phosphate (G3P), the junctional metabolite for lipid synthesis and energy production [8, 9]. Thus, the expression of GK is the prerequisite for cellular glycerol utilization. To our knowledge, little is known about the expression of GK or its mRNA in the oocyte and early embryo.

In the present study, we examined the effect of glycerol on bovine oocyte maturation. Additionally, the expression pattern of GK mRNA was detemined in oocytes before and after maturation and in the embryos at the 2-cell to blastocyst stages.

Materials and Methods

Media

All chemicals were purchased from Wako Pure Chemical (Osaka, Japan), unless indicated otherwise. In the present study, two media were used according to the purpose of the experiment. In the experiment 1, modified synthetic oviduct fluid containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Chemical, St.

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Table 1. Primers used for real-time PCR

Gene	Sequence (5'-3')	Annealing temp. (C)	Size (bp)	Genebank ID No.
Glycerol kinase	S: gtgaaaccatccatgcctga AS: tgtgacagccgacaagtcct	60	111	BC122692
Histone H2AZ	S: ggtaaggctgggaaggactc AS: tcaggtgtcgatgaatacgg	60	103	X52318
GAPDH	S: gagaaggctggggctcacttga AS: cagtgatggcgtggacagtggt	65	232	U85042

S: sense primer, AS: anti-sense primer.

Louis, MO, USA) [13] but devoid of glucose was used as the base medium (mSOF) for *in vitro* maturation (IVM) in order to test the effect of glycerol supplementation on oocyte maturation. In the experiment 2, TCM199 was used for both IVM and IVC in order to obtain sufficient numbers of oocytes/embryos at the different stages required for the gene expression analysis.

Oocyte recovery and IVM

Bovine ovaries were obtained from a local abattoir, and the cumulus-oocyte complexes (COC) were aspirated from small follicles (2 to 5 mm in diameter) using a 5 ml syringe fitted with an 18-gauge needle. COCs with 3 or more layers of cumulus cells and homogenous cytoplasm were selected and washed in the maturation medium.

In the experiment 1, COC were cultured in mSOF containing 0.02 IU/ml of bovine FSH (AFP-5332B, provided by Dr. AF Parlow, NIDDK, Torrance, CA, USA) and 1 μg/ml estradiol (Sigma) supplemented with/without 1.5 mM glucose and/or 1 mM glycerol (Sigma) in a 96-well plate (Nalge Nunc International, Roskilde, Denmark) and cultured for 21 h at 38.5 C in a humidified atmosphere of 5% CO_2 in air (10 oocytes/100 μl). Following IVM, denuded oocytes were fixed in 25% (v/v) acetic acid in ethanol for 24 h and stained in 1% (w/v) orcein in 45% (v/v) acetic acid solution, and the proportion of oocytes at metaphase II (MII) was recorded.

In the experiment 2, groups of 50 COCs were cultured in 500 μl drops of TCM199 with Erle's salts, L-glutamine and 25 mM HEPES (Gibco Invitrogen, Grand Island, NY, USA) supplemented with 5% (v/v) heat-treated fetal bovine serum (HyClone, Logan, UT, USA) and 50 μg/ml gentamicin (Sigma). The COC were cultured for 21 h at 38.5 C in a humidified atmosphere of 5% CO2 in air.

In vitro fertilization (IVF)

IVF was performed according to the method reported by Hamano *et al.* [14]. Briefly, frozen semen from a Japanese Black bull was thawed at 38.0 C for 10 sec and washed twice by centrifugation for 5 min at 800 × g with sperm washing medium. The washed spermatozoa were subsequently suspended in modified Brackett and Oliphant's medium [15] supplemented with 1.25 U/ml heparin (Mochida Pharmaceuticals, Tokyo, Japan) and 5 mM theophylline (Sigma) and adjusted to a final concentration of 3.0×10^6 cells/ml. Expanded COCs were washed in the IVF medium, transferred into IVF drops (50 oocytes/250 μl) and incubated with spermatozoa for 5 h at 38.5 C in a humidified atmosphere of 2% CO_2 in air.

In vitro culture (IVC)

Following IVF, presumptive zygotes were freed from attached cumulus cells by repeated pipetting, transferred to a droplet of maturation medium that had been kept with detached cumulus cells in an incubator and co-cultured for up to 192 h at 38.5 C in a humidified atmosphere of 2% CO_2 in air [14]. At specific time points, oocytes and embryos were collected for gene expression analysis.

Total RNA extraction

Oocytes and embryos were treated with 0.5% (w/v) actinase in PBS containing 0.1% (w/v) PVA and then washed in 0.1% PVA-PBS to remove the zona pellucida. Cumulus cells, oocytes and embryos at each developmental stage were pooled (10 in each group) and homogenized in a denaturing solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl (Sigma), 0.1 M β-mercaptoethanol (Sigma) and 10 μg glycogen per tube to improve RNA recovery. Total RNA was extracted with phenol-chloroform according to the method reported by Chomczynski and Sacchi [16].

Reverse transcription (RT) and quantitative PCR

Removal of genomic DNA and subsequent reverse transcription of RNA was carried out using a QuantiTectρ® Reverse Transcription (RT) kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The abundance of genes encoding GK, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone H2A member Z (H2AZ) was quantified by a real-time PCR (LightCycler; Roche, Basel, Switzerland) using a QuantiTect® SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Each quantification was performed with 1/20 of a cDNA sample (0.5 oocyte equivalent). Primers were designed with Primer3 [17] using reported bovine sequences (Table 1). The amplification program consisted of initial 15 min activation at 95 C followed by 45 cycles of the PCR steps (denaturation at 94 C for 15 sec, annealing at 60 or 65 C for 30 sec and extension at 72 C for 30 sec). For quantification of the target genes, a standard curve was generated using a set of serially diluted DNA fragments that contained the target sequence for real-time PCR. A non-template control set was included in each assay. To monitor genomic DNA contamination, a set of selected non-RT RNA samples was also included. The amplification efficiencies were 1.79 for GK, 1.73 for

Table 2. Effect of glycerol on nuclear maturation of bovine oocytes

Glucose (1.5 mM)	Glycerol (1.0 mM)	No. of oocytes examined	Maturation rate* (%)
−	−	93	13.2 ± 1.3^{a}
+	−	95	45.3 ± 1.6^{b}
−	+	90	7.8 ± 0.5^{a}
+	+	84	30.5 ± 5.0^{c}

*Percentage of oocytes that reached the MII stage at the end of a 21 h IVM period. Values are expressed as the mean ± SEM of 3 replicates. [a,b,c] Values with different superscripts differ significantly ($P<0.05$).

GAPDH and 1.85 for H2AZ. All cDNA samples were amplified in duplicates, and the mean was used for the analysis. The intra- and interassay coefficients of variation were 4.0 and 4.1% for GK, 10.7 and 4.0% for GAPDH and 2.1 and 2.0% for H2AZ, respectively. The GK mRNA abundance was normalized to GAPDH and H2AZ, which have been advocated as better internal controls for bovine, porcine, and murine oocytes and embryos [18–21].

Glucose and lactate assay

The concentrations of glucose and lactate in the medium after IVM were determined by a colorimetric method using commercial kits (glucose, Glucose CII Test; Wako, lactate, Determiner LA; Kyowa Medex, Tokyo). All samples were measured in duplicates, and the mean was used for the analysis. The intra- and interassay coefficients of variation were 2.1 and 3.0% for glucose and 1.6 and 4.1% for lactate, respectively.

Statistical analysis

The frequencies of nuclear maturation in each replicate were arcsine transformed and compared using the Tukey-Kramer multiple comparisons test. The abundance of mRNA was analyzed using the Kruskal-Wallis test followed by the Scheffé's test. Consumption of glucose and production of lactate were analyzed using two-way ANOVA followed by the Tukey-Kramer multiple comparison test. Differences were considered significant at $P<0.05$.

Results

Effects of glycerol on nuclear maturation and expansion of cumulus cells

When the COCs were cultured in medium supplemented with 1 mM glycerol, there was no significant difference in the rate of maturation compared with no energy supplementation (7.8 *vs.* 13.2%, Table 2). On the other hand, the maturation rate was significantly lower ($P<0.05$) for medium containing glucose with glycerol than for that without glycerol (30.5 *vs.* 45.3%). Cumulus expansion was observed only when glucose was added. Addition of glycerol to the glucose-supplemented medium did not affect the cumulus expansion (Fig. 1).

Effect of glycerol on glucose metabolism in COCs

Glucose consumption and lactate production were unaffected by addition of glycerol to the glucose-supplemented media (Table 3). Very low but appreciable levels of lactate were detected in the group without glucose and glycerol. Addition of glycerol did not alter this value.

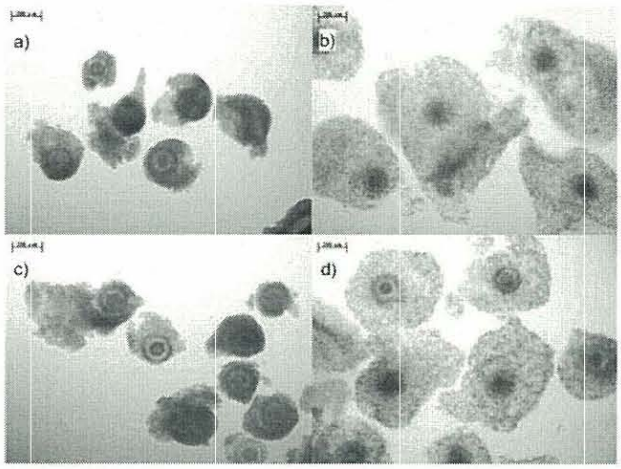

Fig. 1. Morphology of cumulus-oocyte complexes after 21 h-maturation incubation in mSOF supplemented with a) no energy substrates, b) 1.5 mM glucose, c) 1 mM glycerol or d) 1.5 mM glucose and 1 mM glycerol. Bars=200 μm.

Analysis of GK mRNA expression

Transcripts for GK, GAPDH and H2AZ were detected in all RT-samples, whereas no signals were detected in non-RT/non-template controls. The ranges and median cycle threshold (Ct) values were 23.1–36.1 and 28.9 for GK, 14.7–30.3 and 23.3 for GAPDH and 18.1–31.4 and 23.8 for H2AZ, respectively.

The level of GAPDH-normalized GK mRNA expression was low in oocytes (Fig. 2a). Expression of GK mRNA was also low in embryos up to the 2-cell stage, gradually increased by 20-fold in morulae ($P<0.05$) and then decreased in blastocysts (Fig. 3a). The levels of H2AZ-normalized GK mRNA expression were high in oocytes and embryos up to the 2-cell stage and then precipitately decreased at the 8- to 16-cell stages. Increases of more than 10-fold were observed in morulae and hatched blastocysts, but no increase was noted in expanded blastocysts (Fig. 2b and 3b). In the COCs, the abundance of GK mRNA was much lower in cumulus cells than in oocytes regardless of the housekeeping genes used for normalization (Fig. 2a and b).

Table 3. Effect of glycerol on glucose consumption and lactate production in cumulus-oocyte complexes during the IVM period

Glucose (1.5 mM)	Glycerol (1.0 mM)	Glucose consumption (pmol/COC/21 h)	Lactate production (pmol/COC/21 h)
–	–	–	0.1 ± 0.01^{a}
+	–	2.9 ± 0.2	5.5 ± 0.4^{b}
–	+	–	0.1 ± 0.01^{a}
+	+	3.0 ± 0.2	4.3 ± 0.6^{b}

Values are expressed as the mean ± SEM of 10 assays. [a,b] Values with different superscripts differ significantly (P<0.05).

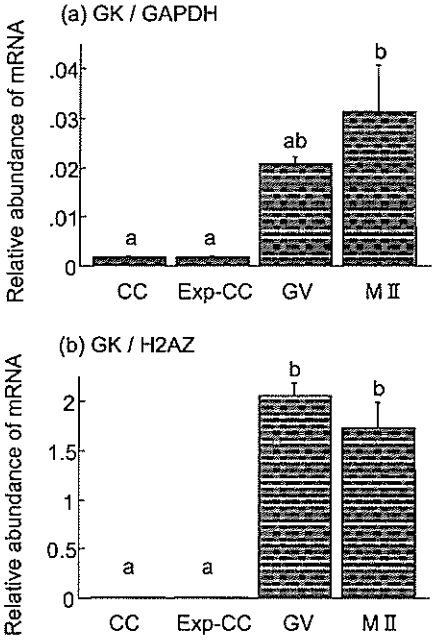

Fig. 2. Relative abundance (mean ± SEM n=3) of GK mRNA normalized to GAPDH (a) or H2AZ (b) in bovine cumulus cells and oocytes before and after the maturation incubation. CC, cumulus cells; Exp-CC, expanded cumulus cells; GV, germinal vesicle oocytes; MII, metaphase II oocytes. a, b: different superscripts indicate significant differences (P<0.05).

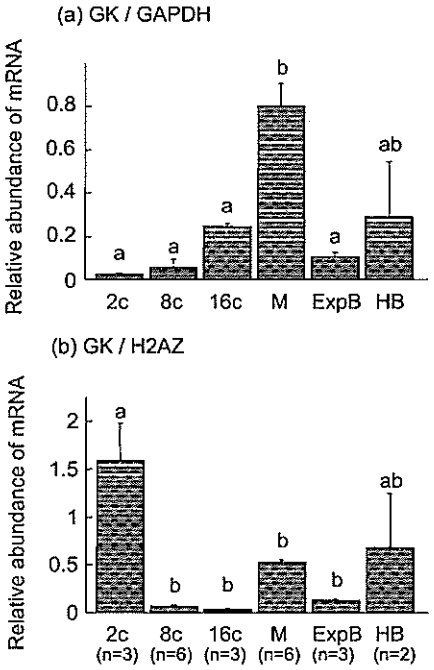

Fig. 3. Relative abundance (mean ± SEM) of GK mRNA normalized to GAPDH (a) or H2AZ (b) in bovine embryos at the 2-cell (2c), 8-cell (8c), 16-cell (16c), morula (M), expanded blastocyst (ExpB) and hatched blastocyst (HB) stages. a, b: different superscripts indicate significant differences (P<0.05).

Discussion

Glycerol plays multi-functional roles in cellular physiology. Other than forming a backbone for glycerophospholipid and triglyceride (TG) synthesis, glycerol acts as an energy substrate for glycolysis [8, 9]. It has been reported that spermatozoa contain enzymes necessary for utilizing glycerol as an energy substrate via the glycolytic pathway [10–12]. However, there are no reports available concerning metabolism of glycerol in the bovine oocyte and preimplantation embryo. Here, we examined the utilization of glycerol as an energy substrate for bovine oocyte maturation and the gene expression pattern of GK, the enzyme crucial for glycerol metabolism and utilization, in bovine oocytes and early embryos.

In the present study, glycerol did not support oocyte maturation, and addition of glycerol to glucose-supplemented media significantly decreased the maturation rate. Although the underlying mechanism of the interference is not clear, the presence of glycerol in culture media may be toxic to the oocyte. It has been reported that exposure to cryopreservation medium containing more than 1.0 M glycerol decreases viability of mouse embryos [22]. In the present study, glycerol was added to a final concentration of 1 mM to test whether it could be used as an energy substrate by the oocyte and embryo. This concentration is much higher than the physiological levels reported in bovine serum (0.03–0.09 mM) [23], although it is very low compared with the level used for study of glycerol metabolism by bull spermatozoa (>10 mM) [11]. These results indicate that as little as 1 mM glycerol interferes with bovine

oocyte maturation. The adverse effect of glycerol is not likely to be caused by alteration of glucose metabolism in cumulus cells because glucose consumption, lactate production and cumulus expansion were unaffected by addition of glycerol in the present study (Table 3).

Low but appreciable levels of lactate were detected when COCs were cultured in medium devoid of glucose (Table 3). This lactate was likely derived from glycogen stored in the cumulus cells [24]. However, it seems that the stored glycogen is not sufficiently utilized for oocyte maturation or cumulus expansion.

GK is the key enzyme that converts glycerol to G3P, which is an intermediary metabolite for synthesis of TG and phospholipids and production of energy. To our knowledge, this is the first report to verifying the expression pattern of GK mRNA in oocytes and embryos. In oocytes and preimplantation embryos, selection of a stable internal control is a troublesome process because the expression of commonly used reference genes fluctuates during oocyte maturation and embryo development [18–20, 25]. Thus, conventional use of a single gene for normalization of both oocytes and embryos may lead to false conclusions. To avoid this problem, normalization by geometric averaging of multiple housekeeping genes is recommended [19, 26]. However, we were unable to take this approach due to lack of a sufficient number of samples. Thus, we used GAPDH and H2AZ as the internal controls on the basis of reports that advocate them as the most stable reference genes in bovine, porcine and murine oocytes and embryos [19–21, 25].

Overall, the expression patterns of GK mRNA were different with the two housekeeping genes. When H2AZ was used as the normalization factor, the GK mRNA expression in oocytes and 2-cell embryos was much higher than that in embryos at advanced stages. This was probably due to very low levels of H2AZ expression in oocytes and 2-cell embryos, as has been reported in the mouse [21]. Conversely, when GAPDH was used as the normalization factor, GK mRNA expression in morulae was higher than those in oocytes and embryos at other stages, except for hatched blastocysts. This discrepancy makes it difficult to compare GK mRNA expression between oocytes/2-cell embryos and embryos at the advanced stages.

Nevertheless, the abundance of GK mRNA was much higher in oocytes than in cumulus cells, regardless of the housekeeping genes used. The lack of GK mRNA expression in cumulus cells might be the reason why the COCs did not utilize glycerol. The expression of GK mRNA in the oocyte implies that glycerol can be metabolized to G3P [9]. It has been reported that the TG concentration in the oocyte prior to maturation is high and that it acts as an energy source during oocyte maturation [27–29]. During this period, lipolytic activity is also high [30]. This may lead to an increase in biosynthesis of intracellular glycerol. Thus, GK may play an important role in utilization of endogenous glycerol as well as exogenous glycerol for synthesis of TG or glycerophospholipid in the bovine oocyte.

Regardless of the housekeeping genes used, expression of GK mRNA in embryos was relatively low during MZT (8- to 16-cells), increased at the morula stage and then decreased at the blastocyst stage. An increase in GK mRNA expression in morulae implies a physiological importance of this enzyme at this stage, in which compaction, a major re-organization of the cellular membrane, occurs. Thus, GK may be necessary for synthesis of phospholipids, which is a major structural component of cellular and intracellular membranes. In the mouse, *de novo* synthesis of phospholipids increases during compaction of the morula [31]. If this is the case in the bovine embryo, the high expression of GK mRNA in morulae might reflect increasing demand for G3P for synthesis of phospholipids.

Although glycerol could not support oocyte maturation under the present experimental conditions, we cannot dismiss possible utilization of glycerol as an energy substrate for the bovine oocyte. As mentioned above, up to 10 pmol/oocyte of glycerol is likely generated as a result of lipolysis during oocyte maturation [27]. Considering the importance of TG as a energy source in oocyte maturation and embryo development [27, 29], it is not unreasonable to assume that glycerol is utilized as an energy substrate along with fatty acids in oocytes and embryos. Further studies are necessary to elucidate these possibilities.

In conclusion, glycerol is not beneficial as a constituent of medium for bovine oocyte maturation. The appreciable levels of GK mRNA found in the oocyte and embryo, however, imply a physiological role for glycerol as a backbone material for essential molecules such as TG and phospholipid.

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