

Effect of Adding Glucose to Maturation Medium on the Nuclear Maturation and ATP Content of Porcine Oocytes

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Abstract: In this study, we investigated the effect of adding glucose to *in vitro* maturation (IVM) medium on the nuclear maturation and ATP metabolism of cumulus cell-enclosed porcine oocytes (COs). When various concentrations of glucose (0.00, 2.78, 5.55, 11.10 and 16.65 mM) were added to IVM medium, the nuclear maturation rate up to the metaphase stage of the second meiotic division (M-II stage) in the 2.78 mM group (67.90%) was significantly higher than that of the non-glucose (0.00 mM, 31.66%) group ($p < 0.05$). In addition, when cumulus cell-denuded oocytes (DOs) were matured in medium with or without 2.78 mM glucose, the rate of DOs maturing to the M-II stage with glucose (53.17%) was significantly higher ($p < 0.05$) than that in the group in which glucose was not added (23.53%). However, these values for DOs were significantly lower ($p < 0.05$) than those for COs (with glucose: 71.85% and without glucose: 38.10%). Conversely, the ATP content of COs and DOs matured with glucose were almost the same as those matured without glucose. These results indicate that glucose may stimulate nuclear maturation without changing ATP metabolism.

Key words: ATP, Glucose, Nuclear maturation, Porcine oocytes

Introduction

Energy substrates such as glucose and pyruvate are necessary for *in vitro* maturation of porcine oocytes [1, 2]. Glucose is metabolized via the glycolysis and/or pentose phosphate pathways and it plays a key role in the control of the nuclear and cytoplasmic maturation of porcine oocytes *in vitro* [3]. Therefore, *in vitro*

maturation (IVM) media such as TCM-199, NCSU23 and NCSU37 [1, 4], used for the culture of porcine oocytes, typically contain glucose at concentrations in the range of 5.55 to 5.56 mM.

Adenosine triphosphate (ATP) is necessary for several cellular functions including motility, maintenance of homeostasis, and regulation of cell survival [5]. ATP is an indicator of oocyte developmental capacity after *in vitro* fertilization in humans and cattle [6–8]. In the mouse, the ATP content of the oocytes is dependent upon the addition of glucose to the maturation medium [9]. Despite these reports, no study has been conducted investigating ATP content and porcine oocyte maturation with or without glucose in the medium.

The present study was undertaken to investigate the relationship between nuclear maturation of porcine oocytes and their ATP content.

Materials and Methods

In vitro maturation

Porcine ovaries were obtained from a local slaughterhouse and immersed in physiological saline (0.9% NaCl; 25–32°C) supplemented with 400 U/ml penicillin G potassium salts (No. 26239-42; Nacalai Tesque, Kyoto, Japan) and 500 µg/ml streptomycin sulfate (No. 32237-72; Nacalai Tesque). The oocytes were aspirated from follicles (2 to 6 mm in diameter) with an 18-gauge needle (No. NN-1838R; Terumo, Tokyo, Japan) attached to a 5 ml syringe. The syringe contained a small amount of TCM-199 (Hank's salt, No. M-0393; Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 2% (v/v) heat inactivated calf serum (CS; 56°C for 30 minutes; No. 16170-086; Gibco BRL Products, Grand Island, NY, USA). Oocytes that were

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enclosed in more than one layer of cumulus cells (COs) were selected and washed four times with TCM-199 without CS. These COs were matured with bovine serum albumin and glucose-free NCSU37 medium [4] for 42 to 46 hours under 5% CO₂, 95% room air and 100% humidity at 39°C. The NCSU37 medium used for IVM was modified with 51.5 mM taurine (No. T-7146; Sigma-Aldrich Co.), 1.2 mM cysteine (No. 10309-12; Nacalai Tesque), 100 μM β-mercaptoethanol (No. M-7522; Sigma-Aldrich Co.), 1% MEM essential amino acid (No. 11130-051; Gibco BRL Products), 0.5% non-essential amino acid (No. 11140-050; Gibco BRL Products), 15 ng/ml epidermal growth factor (No. E-4127; Sigma-Aldrich Co.), 5 μg/ml insulin (No. I-6634; Sigma-Aldrich Co.), 0.23 μg/ml LH (No. L-9773; Sigma-Aldrich Co.), 0.63 μg/ml FSH (No. F-2293; Sigma-Aldrich Co.), 5% (v/v) heat inactivated fetal calf serum (FBS; No. 16140-063; Gibco BRL Products), 100 U/ml penicillin G potassium (No. P-4687; Sigma-Aldrich Co.), 100 μg/ml streptomycin sulfate (No. S-1277; Sigma-Aldrich Co.), and 100 μg/ml dibekacin sulfate (No. DBK; Meiji Seika Co., Tokyo, Japan).

[Experiment 1]: Nuclear maturation assay of the oocytes matured with various concentrations of glucose

The COs collected from 5 replicates (approximately 60 COs/replicate) were matured in modified NCSU37 medium (described above) supplemented with 0.00, 2.78, 5.55, 11.10 or 16.65 mM glucose (No. 16806-25; Nacalai Tesque), zero, 0.5, one, two and three times the usual concentration of glucose (5.55 mM) found in commonly-used maturation media [4]. The media volume during maturation was 5 μl/CO for each group. After IVM, the cumulus cells of the COs were mechanically denuded by a vortex mixer. The oocytes were fixed with acetic alcohol (alcohol : acetic acid = 3 : 1; v/v) and stained with 1% acetic orcein (No. 7100; Merck, Darmstadt, Germany).

[Experiment 2]: Nuclear maturation assay of the oocytes matured with or without cumulus cells with or without 2.78 mM glucose

The COs collected from 6 replicates were randomly divided into two groups. For one group, the COs (approximately 70 COs/replicate) were matured in medium supplemented with or without 2.78 mM glucose in 5 μl media/CO. For the other group, the cumulus cells were removed from the COs (approximately 70 COs/replication) by a vortex mixer (cumulus cell-denuded oocytes; DOs), and were matured in medium supplemented with or without 2.78 mM glucose in 5 μl

media/DO, the same volume as used for the CO groups. After IVM, one aliquot from both the CO and the DO groups (approximately 50 COs and DOs/replicate, respectively) were fixed and stained for evaluation of nuclear maturation. The remaining aliquots from both groups were used to measure the ATP content of each group.

[Experiment 3]: ATP assay

The COs matured with or without 2.78 mM glucose in four replicates had their cumulus cells denuded by a vortex mixer for a few seconds. Subsequently, they were washed twice with physiological saline supplemented with 2% CS. The oocytes in both groups were transferred to 1.5 ml Eppendorf tubes (No. A-150; Assist Co., Tokyo, Japan) with 50 μl of physiological saline supplemented with 2% CS plus 400 μl of distilled water. The solution was then boiled at 100°C for four minutes in a hot dry bath (No. HDB-1; As One Co., Osaka, Japan) and stored at -40°C until assay of the ATP content was conducted. The 2 mM ATP stock solution was thawed and diluted with distilled water to concentrations of 50 to 100 pmol/ml. The 400-μl samples and the ATP stock solution were loaded into a lumicounter (No. A-237; Advantec Co., Tokyo, Japan). Then, to measure the ATP content, purified luciferase (No. 60311; Kikkoman, Chiba, Japan) was added. To measure the ATP content of the immature and DOs matured with or without 2.78 mM glucose in 6 replicates, a similar procedure was used.

Statistical analysis

The percentage of the oocytes that developed to the M-II stage in each group and the ATP content of those oocytes were analyzed by Duncan's multiple range test.

Results

The percentages of the oocytes that developed to the M-II stage in the IVM medium supplemented with various concentrations of glucose are presented in Table 1. Of the 108 immature oocytes collected in five replicates, 83.38% were in the germinal vesicle stage. The percentages for the 2.78 and 5.55 mM groups were significantly higher than that of the non-glucose (0.00 mM) group ($p < 0.05$). The percentage for the 2.78 mM group was the highest of all the groups.

For the oocytes matured with or without cumulus cells, the nuclear maturation rates up to the M-II stage are presented in Table 2. For the COs, the percentage of the oocytes matured with 2.78 mM glucose that

Table 1. Effects of various concentrations of glucose added to the maturation medium on the nuclear maturation of porcine oocytes

Glucose concentration (mM)	N	No. (%) of matured oocytes
0.00	268	84 (31.66 ± 2.37) ^d
2.78	255	176 (67.90 ± 5.19) ^a
5.55	273	178 (64.60 ± 2.18) ^{ab}
11.10	275	143 (51.64 ± 6.54) ^{bc}
16.65	266	130 (48.44 ± 4.50) ^c

^{a, b, c, d}: Values with different superscripts in a column differ significantly ($p < 0.05$). Values are mean ± SE.

Table 2. Effects of glucose on the nuclear maturation of oocytes matured with or without cumulus cells

Treatment	N	No. (%) of matured oocytes
COs –	303	113 (38.10 ± 5.58) ^c
COs +	312	224 (71.85 ± 0.92) ^a
DOs –	341	79 (23.53 ± 5.33) ^d
DOs +	313	166 (53.17 ± 3.28) ^b

^{a, b, c, d}: Values with different superscripts in a column differ significantly ($p < 0.05$). Values are mean ± SE. +; presence of 2.78 mM glucose, –; absence of glucose (0.00 mM glucose).

Table 3. Effects of glucose added to the IVM medium on the ATP contents of the oocytes matured with or without cumulus cells

Treatment	ATP content of the oocytes (p mol/CO and DO)
Immature	0.97 ± 0.14 ^b
COs –	1.35 ± 0.25 ^a
COs +	1.44 ± 0.01 ^a
DOs –	0.39 ± 0.05 ^c
DOs +	0.50 ± 0.09 ^c

^{a, b, c}: Values with different superscripts in a column differ significantly ($p < 0.05$). Values are mean ± SE. +; presence of 2.78 mM glucose, –; absence of glucose (0.00 mM glucose).

reached the M-II stage after IVM was significantly higher than that of the COs matured without glucose ($p < 0.05$). For the DOs matured with 2.78 mM glucose, the percentage that reached the M-II stage was significantly higher than that of the DOs matured without glucose ($p < 0.05$). However, the values in both DO groups were significantly lower than those of both CO groups ($p < 0.05$).

The ATP content of the oocytes in each group are presented in Table 3. Although the ATP content of the oocytes derived from COs matured with or without 2.78 mM glucose was significantly higher than that of the immature oocyte group ($p < 0.05$), there was no significant difference between both CO groups after IVM with or without glucose. For DOs matured with or without 2.78 mM glucose, the ATP content was significantly lower than that of immature oocytes group ($p < 0.05$). However, like the CO groups, when the ATP content of DOs matured with or without glucose was compared, no significant difference was observed.

Discussion

COs matured with both 2.78 and 5.55 mM glucose exhibited high nuclear maturation rates up to the M-II stage and these were significantly higher than that of

the non-glucose group ($p < 0.05$). This finding indicates that the addition of 2.78 to 5.55 mM of glucose to the maturation medium was suitable for the nuclear maturation of the oocytes. Previous studies reported that the glucose concentration of the porcine follicular fluid freshly collected from oocytes 3 to 6 mm in diameter (the same size as those used in the present study), ranged from 2.1 to 4.8 mM up to the preovulatory stage [10, 11]. Our study found that concentrations similar to follicular glucose were optimal for the nuclear maturation of the oocytes.

The ATP content from the oocytes derived from COs matured with or without glucose were significantly higher than that of the immature oocytes. These finding are comparable to those of a previous study [12]. Conversely, the ATP content of both DO groups matured with or without glucose was less than that of the immature group. In our preliminary study, the survivability assessed by 1.7% Eosin-Y staining [13] of the DOs matured with glucose was 78.68%; the percentage of those matured without glucose was 74.72%; these percentages were not significantly different from those of the immature group (76.78%). Thus, the ATP decrease observed in DO groups after IVM in the present study may not be caused by a

decrease in the survival rate of the oocytes during maturation. In cattle, cumulus cells require more glucose than the oocytes during IVM [14]. This phenomenon may cause variance in the ATP content of the oocytes matured with or without cumulus cells after IVM.

In the present study, both COs and DOs, cultured with 2.78 mM glucose, had increased maturation rates up to the M-II stage when compared with those cultured without glucose. In the mouse, glucose is metabolized to pyruvate by cumulus cells; thus, promoting the nuclear and cytoplasmic maturation of the oocytes [15]. Downs *et al.* [16] suggested that mouse DOs can use not only pyruvate but also glucose for IVM. Increase of the maturation rate of DOs cultured with glucose, in the present study, indicates that porcine DOs may also utilize glucose during IVM.

Although the nuclear maturation rates from the CO and DO groups matured with 2.78 mM glucose were significantly higher than those in both groups matured without glucose ($p < 0.05$), the ATP content of the oocytes derived from COs matured with 2.78 mM glucose was the same as that of the COs matured without glucose; this finding was repeated in the DO groups. This indicates that glucose can accelerate nuclear maturation up to the M-II stage without changing the ATP content. Downs [9] noted that glucose-free maturation medium decreased nuclear maturation and ATP content of mouse oocytes. Although the similar ATP content of oocytes matured with or without glucose in the present study is inexplicable, some hypotheses can be proposed.

It is possible that porcine oocytes may use an intracellular lipid as an energy substrate for maturation [17, 18]. Since the oocytes derived from COs or DOs matured without glucose, the lipid droplets might be used for ATP production, substituting for glucose; this would result in maintenance of the ATP content at the oocyte levels of both CO and DO groups matured with glucose. FBS usually contains approximately 6.66 mM of glucose [19]. In view of this, our maturation medium which was supplemented with 5% FBS contained approximately 0.33 mM glucose; thus, the final concentrations of glucose in the non-glucose group was 0.3 mM and 3 mM in the 2.78 mM group. The ATP of the oocytes matured without additional glucose supplementation may have been produced via the utilization of the low concentration of glucose in the 0.00 mM group; however, this low level concentration of glucose could not support the nuclear maturation of the oocytes.

In conclusion, glucose can accelerate the nuclear maturation without changing the ATP content of porcine oocytes during IVM.

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