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The effect of high glucose concentration on the quality of oocytes derived from different growth stages of follicles

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Abstract: This study examined the effect of hyperglycemic culture conditions on the development of oocytes derived from early antral follicles (EAFs). Oocyte–granulosa cell complexes (OGCs) derived from EAFs were cultured for 12 days in medium containing 5.56 mM or 11 mM glucose, and the rate of antrum formation and glucose consumption by the OGCs, and the characteristics of the oocytes grown *in vitro* were studied. The results were compared with those obtained from *in vivo* grown oocytes derived from antral follicles (AFs; 3–5 mm in diameter). In addition, the effect of a high glucose concentration in the maturation medium on the quality of oocytes derived from AFs was examined. The high glucose condition increased the glucose consumption of the OGCs but did not affect antrum formation, oocyte diameter, chromatin configuration, levels of histone 4 K12 acetylation, nuclear maturation, and the developmental ability of oocytes grown *in vitro*. In contrast, high glucose-maturation medium increased the amount of reactive oxygen species and adversely affected the developmental ability of the oocytes. In conclusion, the results of this study suggest that culture under hyperglycemic conditions is detrimental to oocyte maturation but not for oocyte growth from the EAF stage to the AF stage.

Key words: Porcine, OGCs, IVG, Glucose, Diabetes

Introduction

Maternal diabetes and hyperglycemia adversely affects maternal reproductive functions [1, 2]. In animal studies, it has been reported that the quality of oocytes obtained from streptozotocin-induced diabetic mice is low: with ovulated oocytes displaying delayed meiotic maturation progress, abnormal spindle formation, and mitochondrial dysfunction [3, 4]. In addition, the oocytes have been reported to be small, and the percentage of apoptotic granulosa cells was high [5]. Furthermore, although histone 4 K12 (H4K12) acetylation levels in germinal vesicle (GV) stage oocytes are generally higher than those in MII stage oocytes [6, 7], they were found to be low in oocytes from streptozotocin-induced diabetic mice [5]. However, the etiology of the abnormal oocytes derived from diabetic animals has not been clarified, as hyperglycemia induces multiple physiological changes. Moreover, an adverse effect of high glucose during oocyte maturation on the quality of oocytes derived from antral follicles has been reported in cows [8]. Hence, the aim of the present study was to examine the effect of hyperglycemic culture conditions on the quality of oocytes from oocyte–granulosa cells complexes (OGCs) derived from early antral follicles (EAFs) and grown *in vitro*. The quality of the oocytes was assessed by examining oocyte size, chromatin configuration, levels of H4K12 acetylation, nucleic maturation, and the ability of the resulting embryos to develop to the blastocyst stage. In addition, we examined the effect of using hyperglycemic medium during *in vitro* maturation (IVM) on the quality of *in vivo* grown oocytes derived from antral follicles (AFs), as assessed by nucleic maturation, the ability of the resulting embryos to develop to the blastocyst stage, and the levels of reactive oxygen species (ROS).

Materials and Methods

Drugs and media

All drugs were purchased from Nacalai Tesuque (Kyoto, Japan) unless otherwise stated. *In vitro* culture of growing porcine oocytes was performed using α-minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA)

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supplemented with 10 mmol/L taurine, 0.1 mAU/ml FSH (Kawasaki Mitaka, Tokyo, Japan), 1 mg/ml estradiol-17*β*, 2% polyvinylpyrrolidone-360 (Sigma-Aldrich), 2 mmol/L hypoxanthine (Sigma-Aldrich), 1% ITS-G (Gibco BRL, Paisley, UK), 3 mg/ml BSA (Fraction-V), and antibiotics. *In vitro* maturation was performed using North Carolina State University 23 medium supplemented with BME Amino Acid Solution (Sigma-Aldrich), MEM Non-essential Amino Acid Solution (Sigma-Aldrich), and 10% porcine follicle fluid (FF). The FF was collected from antral follicles (3–5 mm in diameter), centrifuged (10,000×*g*, 5 min, 4 °C) and stored at –20 °C until use. The culture media used for embryo development were porcine zygote medium-3 (PZM-3) containing 3 mg/ml BSA (fatty acid free) and PZM-3 in which BSA had been replaced with 3 mg/ml polyvinyl alcohol (PZM-4) [9].

Glucose concentration in medium

The glucose concentration in FF closely reflects that in serum [10], and is slightly less than the serum glucose concentration in both cows (2.0 and 5.1 mM) [11] and pigs (4.1 and 5.9 mM) [12], respectively. According to the WHO, a plasma glucose concentration >11.1 mM is the criterion for diagnosing diabetes [13]. Therefore, based on this criterion, 11 mM of glucose was used as the high glucose condition, and 5.56 mM was used as the normal glucose concentration.

Animal and collection of ovaries and oocytes

Ovaries were obtained from prepubertal gilts at a local abattoir and transported in PBS containing antibiotics at 37 °C to the laboratory within 1 h. Cumulus cell–oocyte complexes (COCs) were aspirated from AFs (3–5 mm in diameter) using an 18-gauge needle connected to a 10 ml syringe. Subsequently, the ovarian surface was sliced, and OGCs were collected from EAFs (0.5–0.7 mm in diameter) by using precision tweezers and a 21-gauge needle that was connected to a 1 ml syringe. Oocytes that were surrounded by a thick and compact layer of somatic cells were selected for use in the subsequent experiments.

In vitro culture of OGCs

In vitro culture of OGCs was conducted as previously described [14]. Briefly, OGCs were individually cultured in 200 *μ*l of medium in a 96-well plate (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) for 12 days at 38.5 °C with 5% CO_2 and maximum humidity. Half of the medium was replaced with fresh medium at 4-day intervals, at which time antrum formation was examined. At the end of the culture period, OGCs that had undergone antrum formation were selected, and the oocytes and surrounding cells were retrieved from the OGCs by using precision tweezers and a 21-gauge needle that was connected to a 1 ml syringe. These oocytes were then used for further experiments.

In vitro maturation of oocytes

In vitro maturation was conducted as previously described [15]. Cumulus cell–oocyte complexes collected from AFs or oocytes surrounded by cells retrieved from OGCs that had developed *in vitro* were cultured in maturation medium (1 oocyte/10 *μ*l) at 38.5 °C with 5% CO_2 and maximum humidity. For the first 20 h, maturation medium containing 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich) and hormones (10 IU/ml equine chorionic gonadotropin, and 10 IU/ml human chorionic gonadotropin) was used. The COCs were then transferred to a maturation medium without dbcAMP and hormones and cultured for a further 24 h. In order to examine nuclear maturation, the oocytes were fixed and mounted on a slide glass with a ProLong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, CA, USA), and were observed under a fluorescence digital microscope (BZ-8000; Keyence, Tokyo, Japan).

Determination of glucose concentrations in medium

We classified OGCs based on their viability: OGCs which had undergone antrum formation by the end of the culture period (day 12) were defined as viable OGCs, and those that had not were defined as nonviable OGCs. The culture medium was collected when the medium was changed (days 4 and 12), because antrum formation occurs from day 4 to day 6. These samples were then transferred to a test tube and stored at −20 °C until examination. The glucose concentrations were measured using a Spotchem-SE electrolyte analyzer (Arkray Inc., Kyoto, Japan). Seventeen samples were randomly selected from the normal glucose group (viable OGCs, n=10; nonviable OGCs, n=7), and 16 samples were randomly selected from the high glucose group (viable OGCs, n=10; nonviable OGCs, n=6).

Oocyte diameter and chromatin configuration

Oocyte diameter was measured under a fluorescence digital microscope (BZ-8000) and calculated using *x* and *y* coordinates. In order to examine the chromatin configuration of the oocytes, the oocytes were fixed with acetic acid/ethanol (1:3, v/v) for 1 week and then stained with 2% aceto-orcein. The chromatin configuration was classified into five groups as described in previous studies [16–18] with slight modifications, as follows: GV0, noncondensed

chromatin; GV1, slightly condensed chromatin which is stained only around the nucleolus; GV2, a few chromatin clumps appear near the nuclear membrane; GV3, condensed chromatin clumps or strands were distributed throughout the nucleoplasm; and GV4, condensed chromatin clumps or strands were present, but the nuclear membrane was not distinct and the nucleolus had completely disappeared.

Immunostaining of H4K12 acetylation

Immunostaining was conducted as previously described [19]. The primary and secondary antibodies used were a rabbit polyclonal anti-acetyl H4K12 (1:500; Millipore, Milford, MA, USA) and Anti-rabbit IgG (H + L), F $(ab')_2$ Fragment (Alexa Fluor 555 Conjugate; 1:1000; Cell Signaling Technology Inc., Beverly, MA, USA), respectively. The oocytes were mounted on glass slides under an antifade reagent containing DAPI (Invitrogen) and were observed using a fluorescence digital microscope (BZ-8000). Fluorescence images of the oocytes were captured, and the fluorescence intensities of the GVs were measured using ImageJ software (NIH, Bethesda, MD, USA).

Reactive oxygen species measurement

Oocyte ROS levels were measured using ROS Detection Reagents (Invitrogen) as previously described [20]. The fluorescence intensities of the oocytes were examined under a fluorescence digital microscope (BZ-8000), and the pixel intensity was transformed using ImageJ software.

Activation and in vitro culture of embryos

For IVM, oocytes were denuded of surrounding cells and activated by incubation in PZM-3 containing 10 μg/ml ionomycin for 5 min followed by culture for 6 h in medium containing 10 μg/ml cytochalasin B and 10 μg/ml cycloheximide. After activation, the embryos were cultured in PZM-4 for 8 days, and then the rate of blastulation and the cell number of the resulting blastocysts was determined. The *in vitro* culture was conducted at 38.5 °C with 5% CO_2, 5% O_2, 90% N_2, and maximum humidity.

Experimental design

Assessing the effect of glucose concentration on oocytes from cultured EAF OGCs

Approximately 15 OGCs derived from EAFs were cultured in medium containing 5.56 mM or 11 mM glucose for 12 days, during which time the rate of antrum formation was examined. This experiment was performed 25 times. The first eight times, chromatin configuration and oocyte diameter were assessed in the resulting oocytes. In oocytes from the next two replicates, the levels of H4K12 acetylation were examined by immunostaining. Oocytes grown in the following eight replicates were subjected to IVM, after which the rate of nuclear maturation was examined. Oocytes from the last seven replicates were matured and activated, and then the rate of blastulation was assessed 8 days after activation. When examining oocyte diameter, chromatin configurations, and levels of H4K12 acetylation, fresh oocytes obtained from EAFs and AFs were used as the control. In addition, glucose concentrations were measured in samples of harvested medium in order to calculate OGC glucose consumption.

Assessing the effect of glucose concentration on oocytes matured in vitro

Twenty oocytes were matured in IVM medium containing 5.56 mM or 11 mM glucose. The experiment was performed 16 times. In 6 replicates, the nuclear maturation rate was assessed. In 4 replicates, the ROS levels were measured. In 6 replicates, the rate of development to the blastocyst stages was assessed.

Statistical analysis

Data were compared using ANOVA followed by the Tukey's post-hoc test. A *P*-value less than 0.05 was considered to indicate significance. The antrum formation rate, percentage of chromatin configuration, nuclear maturation rate, and developmental rate were arc sine transformed before the analysis.

Results

High glucose increased OGC glucose consumption

First, we addressed whether the glucose concentration in the culture medium affects OGC glucose consumption. As seen in Fig. 1, consumption of glucose significantly increased during culture periods between day 4 and day 12 of culture, and consumption was higher by OGCs cultured in high glucose medium than by those cultured in normal glucose medium (high glucose: 0.7 mM on day 4 vs. 3.1 mM on day 12; normal glucose: 0.4 mM on day 4 vs. 2.3 mM on day 12; $P < 0.05$). In addition, by day 12, glucose consumption by viable OGCs was significantly greater than that by nonviable OGCs (normal glucose: 2.3 mM for viable OGCs vs. 0.8 mM for nonviable OGCs; high glucose: 3.1 mM for viable OGCs vs. 0.7 mM for nonviable OGCs; $P < 0.05$), although no difference could be seen at day 4.

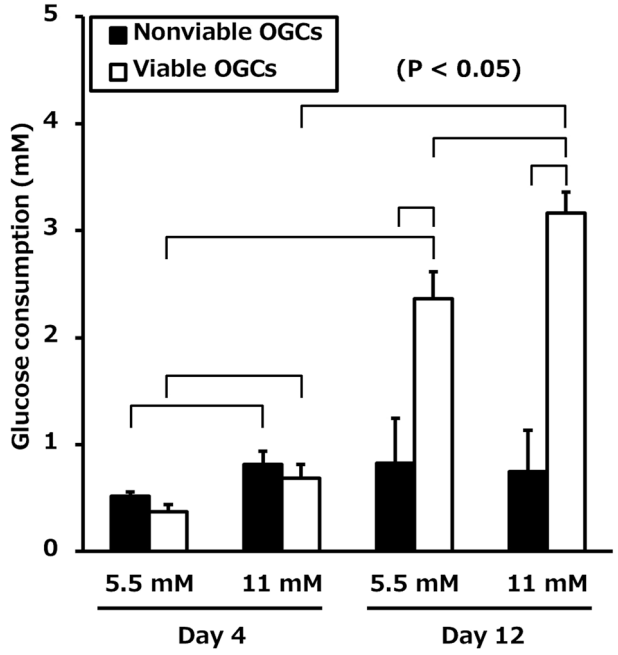

Fig. 1. Glucose consumption of oocyte–granulosa cell complexes (OGCs) cultured in medium containing 5.5 mM or 11 mM glucose for 4 or 12 days. Oocyte–granulosa cell complexes were categorized based whether they had undergone antrum formation (viable or nonviable) by the end of the culture period; brackets indicate pairs that were significantly different (P <0.05).

The rates of antrum formation in OGCs cultured in high glucose and those cultured in normal glucose were not significantly different

Antrum formation is a marker of developmental competence in OGCs. As seen in Fig. 2, antrum formation started between day 4 and day 6 of culture. By the end of the culture period, 69.0% of OGCs cultured in normal glucose and 75.1% of OGCs cultured in high glucose had showed undergone antrum formation; however, the difference between these percentages was not significant.

Chromatin condensation increased during in vitro development

The chromatin configuration of all oocytes from EAFs was categorized as GV0 upon collection; however, the percentage of oocytes with GV0 chromatin plummeted during oocyte development, concomitant with an increase in the percentage of oocytes with GV1 chromatin to 48.5% and 62.0% for OGCs cultured under normal and high glucose, respectively (Table 1). The distribution of chromatin configurations was similar for the *in vivo* developed oocytes and the two glucose concentration groups. Histone acetylation increased strongly as oocytes developed, but the level of H4K12 acetylation did not differ between oocytes from OGCs cultured in high glucose and those from OGCs cultured in normal glucose (Fig. 3). However, in both groups, the level of acetylation did not reach the level seen in oocytes that had developed *in vivo*.

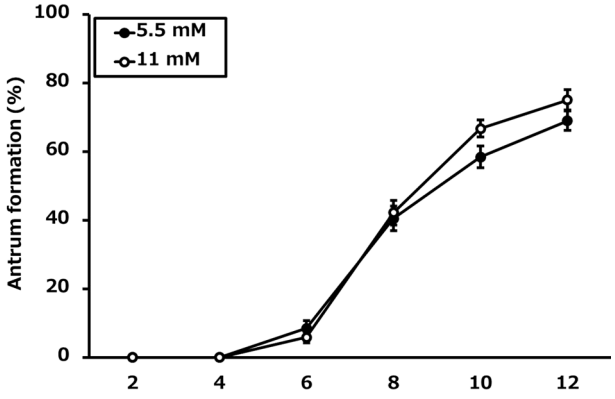

Fig. 2. Percentage of oocyte–granulosa cell complexes that displayed antrum formation following culture in medium containing 5.5 mM or 11 mM glucose.

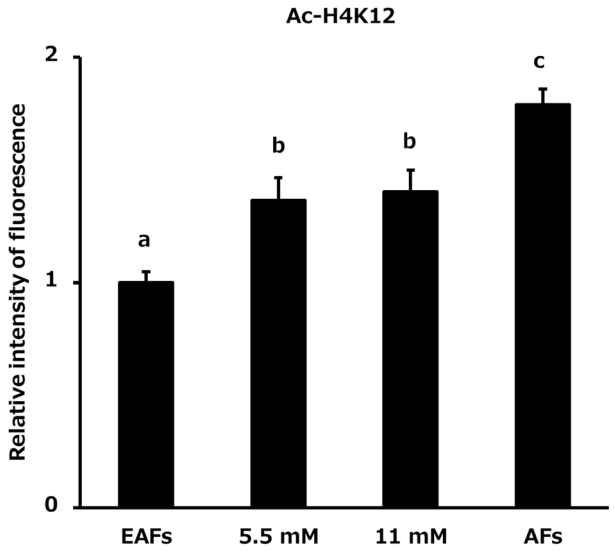

Fig. 3. Levels of histone 4 K12 acetylation (Ac-H4K12) in oocytes collected from early antral follicles (EAFs), oocytes collected from antral follicles (AFs), and oocytes collected from EAFs and cultured in medium containing 5.5 mM or 11 mM glucose. The fluorescence intensity of the oocytes collected from EAFs was defined as 1.0; Different letters indicate a significantly difference (P <0.05).

Among the OGCs cultured under normal or high glucose concentrations, 50.8% and 56.5% of the oocytes

Table 1. Effect of glucose in *in vitro* growth medium on the GV chromatin configuration of oocytes

Experimental groups	No. of oocytes examined	oocytes diameter (Mean ± SEM, μm)	Rate of chromatin configuration (Mean ± SEM, %)			Rate of oocytes degenerated (Mean ± SEM, %)
			GV0	GV1	GV2–4	
EAFs	64	97.6 ± 0.5[a]	100[b]	0[a]	0[a]	0[a]
AFs	69	121.6 ± 0.9[c]	4.2 ± 0.4[a]	59.5 ± 5.6[b]	36.3 ± 6.0[b]	0[a]
5.5 mM	68	112.4 ± 1.0[b]	11.6 ± 5.4[a]	48.5 ± 7.9[b]	32.2 ± 9.6[b]	7.7 ± 4.1[b]
11 mM	73	114.6 ± 0.9[b]	6.5 ± 3.4[a]	62.0 ± 4.5[b]	29.4 ± 2.7[b]	2.1 ± 1.4[b]

GV, germinal vesicle; EAFs, early antral follicles; AFs, antral follicles. [a–c];Within a column, means without a common superscript letter differed ($P < 0.05$).

Table 2. Effect of glucose in *in vitro* oocyte growth medium on the nuclear maturation of oocytes

Experimental groups	No. of oocytes examined	Rate of nuclear maturation (Mean ± SEM, %)					Rate of oocytes degenerated (Mean ± SEM, %)
		GV	GVBD	MI	AT	MII	
AFs	54	0[a]	0	5.6 ± 0.2[a]	4.3 ± 1.6	90.1 ± 1.8[b]	0
5.5 mM	86	19.6 ± 3.8[b]	2.2 ± 1.2	17.2 ± 4.0[b]	8.2 ± 2.3	50.8 ± 5.8[a]	2.0 ± 1.6
11 mM	98	19.2 ± 2.3[b]	0.8 ± 0.6	8.7 ± 2.1[a]	12.8 ± 2.7	56.5 ± 3.1[a]	0.9 ± 0.7

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AT, anaphase I and telophase I; MII, metaphase II; AFs, antral follicles. [a,b]; Within a column, means without a common superscript letter differed ($P < 0.05$).

Table 3. Effect of glucose in *in vitro* growth medium on the embryo development of oocytes

Glucose (mM)	No. of oocytes examined	No. of blastocysts (Mean ± SEM, %)	No. of cells (Mean ± SEM)
5.5	74	3 (4.8 ± 2.4)	54.0 ± 14.7
11	87	4 (4.9 ± 3.9)	34.3 ± 10.7

Table 4. Effect of glucose in *in vitro* oocyte maturation medium on nuclear maturation and development to the blastocyst

Glucose (mM)	No. of trials	No. of oocytes	Maturation (Mean ± SEM, %)		No. of trials	No. of oocytes	Blastulation (Mean ± SEM, %)	Cell No. (Mean ± SEM)
			Not Matured	MII				
5.5	6	120	19.8 ± 4.1	80.2 ± 4.1	6	120	21.1 ± 4.1	46.7 ± 3.1[a]
11	6	120	18.9 ± 1.7	81.1 ± 1.7	6	120	11.7 ± 3.3	36.1 ± 2.9[b]

MII, metaphase II. [a,b]; Within a column, means without a common superscript letter differed ($P < 0.05$).

reached the MII stage, respectively, and the rate was significantly lower than that of the oocytes that had developed *in vivo* (90.1%, Table 2). When oocytes grown *in vitro* under high or normal glucose concentrations were activated, similar percentages of oocytes developed to the blastocyst stage (Table 3); however, the rates were very low compared with that of oocytes collected from AFs (21.1%, Table 4).

Hyperglycemia during IVM decreased oocyte quality

Although the rate of nuclear maturation for oocytes matured under high glucose was similar to that of oocytes matured under normal glucose, oocytes matured under high glucose displayed low blastulation rates and blastocyst cell numbers (Table 4). In addition, the ROS levels were higher for oocytes matured under high glucose than for those cultured under normal glucose (relative fluorescence intensity: 1 and 1.17, respectively; $P < 0.05$; Fig. 4).

Discussion

The results of the present study indicates that a high glucose concentration in the culture medium affects

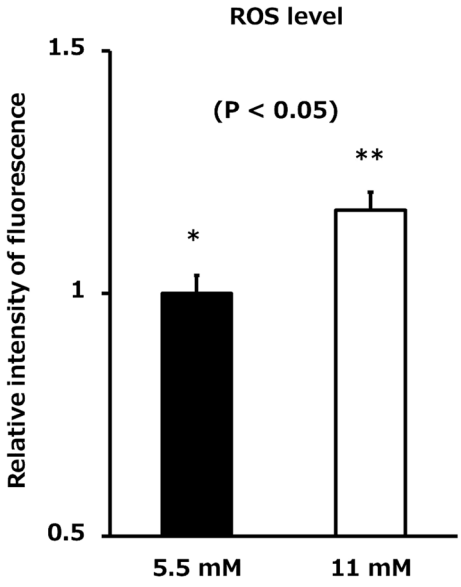

Fig. 4. Amount of reactive oxygen species (ROS) in oocytes matured in medium containing 5.5 mM or 11 mM glucose; **P* <0.05; the fluorescence intensity of the oocytes grown under 11 mM glucose was defined as 1.0.

glucose consumption by OGCs derived from EAFs, although the markers used to predict the developmental competence of the oocytes did not differ between the two glucose concentrations. In addition, the results show that the maturation medium with the high glucose concentration was harmful to the oocytes. Although some reports have shown close links between the glucose concentration of the cell culture medium and glucose metabolism in oocytes during meiotic maturation [21, 22], few have been investigated about the effect of glucose concentrations on OGCs derived from EAFs. In our preliminary experiment, OGCs collected from EAFs had 8,394 ± 552 (n=32) granulosa cells, whereas OGCs having an antrum after 12 days of *in vitro* culture period had of 161,884 ± 37,517 (n=50) granulosa cells. The present results shows there was a significant increase in glucose consumption of OGCs during *in vitro* culture periods indicating the increase in glucose consumption was partly due to the increased cell number of OGCs. They also, the present study shows that although the morphology of the OGCs is almost similar in the two glucose concentrations, the high glucose concentration increased OGCs glucose consumption, thus indicating that the amount of glucose present in the culture medium affects carbohydrate metabolism. Furthermore, the amount of glucose consumption on day 4 did not reflect oocyte developmental ability; this result indicating that glucose consumption at this stage is not a primary factor of further development, although the level of OGC steroid production at day 4 closely reflects the further developmental ability of OGCs in pigs and cows [14, 23].

In a subsequent series of experiments, we examined and compared competence markers for oocytes cultured under a high glucose concentration and those cultured under a normal glucose concentration. The markers were selected based on data from previous reports [18, 24, 25]. When OGCs were cultured in medium containing the high or normal glucose concentrations, similar rates of antrum formation were observed. Antrum formation is a marker of *in vitro* development in OGCs, and the formation and maintenance of antrum structure is closely associated with the degree of interaction between oocytes and the surrounding granulosa cells as well as the viability of both cell populations. Our results indicate that a high glucose concentration does not affect OGC viability. In addition, our results show that the chromatin configurations and levels of H4K12 acetylation in oocytes grown *in vitro* were similar for OGCs cultured under the two glucose concentrations. Moreover, the rate of nuclear maturation and the blastocyst developmental rate were also similar for OGCs cultured under the two glucose concentrations. Given that the level of H4K12 acetylation is low in oocytes derived from diabetic mice [5], our results may indicate that the abnormal histone acetylation observed in the diabetic mice is not caused by a high glucose concentration but by cellular and endocrinal factors associated with diabetes, and that a high glucose concentration during oocyte development from EAFs to AFs does not of itself affect oocyte quality.

It is noteworthy that the percentage of oocytes with chromatin in the GV1 configuration increased during *in vitro* culture and reached a level similar to that seen in oocytes that had developed *in vivo*. However, the observation that the level of H4K12 acetylation in oocytes from cultured OGCs was significantly lower than that observed in oocytes grown *in vivo* indicates that the oocytes grown *in vitro* achieved insufficient growth. This hypothesis is supported by the finding that, for the oocytes grown *in vitro*, the nuclear maturation rate and rate of development to the blastocyst stage were substantially lower than those of the oocytes that had developed *in vivo*. In addition, our results suggest that the acetylation level detects the difference between oocytes that have developed *in vivo* and those that have developed *in vitro* more precisely than the chromatin configuration does.

When the oocytes matured in the medium containing the high glucose concentration were activated, the rate of blastulation and the total cell number of the blastocysts

were lower than the rates of the activated oocytes that had been matured in the medium containing the normal glucose concentrations. In addition, a high glucose concentration in the maturation medium increased the amount of ROS produced by the oocytes. It has been reported that a high glucose concentration induces ROS generation has previously been in reported for somatic cells [26, 27], and the induction of ROS generation by a high glucose concentration in maturation medium has also been demonstrated for bovine oocytes and embryos [8, 28]. Ou *et al.* reported that serum glucose levels were high in hyperinsulinemic mice; in addition, oocytes collected from these mice had high amounts of ROS at both the GV and MII stages, and the developmental competence of the oocytes was low compared with that of oocytes from control mice [29]. Moreover, Chang *et al.* reported that competence of *in vivo* matured oocytes from hyperglycemic mice was low [5]. The molecular mechanism behind the high ROS content in oocytes cultured in high glucose medium is unclear. it is noteworthy that the present culture experiments conducted under a high oxygen concentration (20%), and that the glucose affects carbohydrate metabolism including (oxidative phosphorylation, lipid *β*-oxidation, and glycolysis) [30, 31], and that oxygen tension affects carbohydrate metabolism as well [32, 33]. Therefore, the high O_2 tension may affect high glucose associated ROS generation. In the present study, we did not measure ROS levels in oocytes grown *in vitro*, as there was an insufficient number of oocytes available following the long culture periods. However, taking into consideration the similar developmental rates of the oocytes cultured under the different glucose concentrations, it has been suggested that the toxic effect of a high glucose concentration is more predominant during the maturation period than during the period of growth from the EAF stage to the AF stage. Taken together, these results indicate that high glucose does not affect the *in vitro* growth of oocytes derived from EAFs but does adversely affect oocyte quality during oocyte maturation.

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