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Effect of glucose and pyruvate on nuclear and cytoplasmic maturation of porcine oocytes in a chemically defined medium

Hiroaki Funahashi* Takayuki Koike† Ryoko Sakai‡

*Department of Animal Science, Okayama University, hirofun@cc.okayama-u.ac.jp
†Department of Animal Science, Okayama University
‡Department of Animal Science, Okayama University

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Effect of glucose and pyruvate on nuclear and cytoplasmic maturation of porcine oocytes in a chemically defined medium

Hiroaki FUNAHASHI[1], Takayuki KOIKE[1], and Ryoko SAKAI[1]

[1]Department of Animal Science, Okayama University, Tsushima-Naka, Okayama, 700-8530, Japan

Subtitle: Roles of glucose and pyruvate in porcine IVM

Correspondence should be to H Funahashi; Email: hirofun@cc.okayama-u.ac.jp

Abstract

The present study was undertaken to examine the potential roles of glucose and pyruvate in nuclear and cytoplasmic maturation of porcine oocytes. Oocyte-cumulus complexes (OCC) from 3 to 6 mm follicles were cultured in a chemically defined medium, pyruvate-free mNCSU37-PVA with/without 5.55 mM glucose during in vitro maturation (IVM), germinal vesicle breakdown (GVBD) and nuclear maturation of porcine oocytes were prevented in glucose-free medium ($P<0.05$). Subsequently, OCC were cultured for IVM in glucose-containing mNCSU37-PVA supplemented with various concentrations of 6-amononicotinamide (6-AN) and diphenyleneiodonium (DPI), inhibitors of the pentose phosphate pathway (PPP); both compounds ($\geq$10 μM 6-AN and $\geq$10 nM DPI) inhibited resumption of meiosis ($P<0.05$). Supplementation of glucose-free maturation medium with increasing concentrations of pyruvate induced resumption of meiosis and increased the incidence of oocytes reaching the metaphase-II stage in a concentration-dependent manner ($P<0.05$). More mature oocytes were obtained in the presence of pyruvate + glucose ($P<0.05$). After culture to allow maturation, glutathione content was higher in the oocytes cultured in the presence of pyruvate, alone than in those cultured in glucose alone; inclusion of 6-AN abolished responses to pyruvate ($P<0.05$). These results demonstrate that, in the pig, both glucose and pyruvate play a critical role in the release of oocytes from arrest at the GV-I stage, probably through the PPP, whereas supplementation with pyruvate improves the cytoplasmic maturation as determined by oocyte glutathione level.

1. Introduction

Mammalian oocytes metabolize an essential energy substrate, glucose, via glycolysis, the pentose phosphate pathway (PPP) and the tricarboxylic acid cycle [1-3]. The PPP and/or glycolysis appears to play a critical role in resumption of meiosis in mouse oocytes [4,5]. Gonadotropins induce mouse oocytes to resume via gap junction coupling, between oocyte and cumulus cells [6,7], but only when glucose is present in the maturation medium [8,9]. This resumption of meiosis appears to be associated with elevated activity of glycolysis and the PPP [1,4], but elevated very high levels of glucose have been shown to suppress maturation [9-11]. Furthermore, there appears to be some differences in the glucose requirement for oocyte maturation among mammalian species. In primate oocytes, nuclear maturation can occur in the absence of carbohydrates, but glucose is necessary for cytoplasmic maturation [12]. In bovine oocytes, increased metabolism of glucose through one or more metabolic pathways has been reported to occur simultaneously with the progression of meiosis to the metaphase-II stage [13]; this elevated glucose metabolism in mature oocytes correlates with improved developmental competence of bovine embryos [2]. In porcine oocytes, investigations using a chemically defined glucose-containing medium supplemented with stimulators or inhibitors of glycolysis and the PPP [14] have reported that metabolism through the PPP and/or glycolysis affects the control of nuclear and cytoplasmic maturation in vitro. Sturmey and Leese [15] demonstrated that the triglyceride content of porcine oocytes decreases during maturation, leading them to suggest that a high glucose concentration in the culture medium may be needed to form pyruvate, which in turn, produces oxaloacetate that is required to prime the tricarboxylic acid cycle. However, details regarding the roles of glucose and pyruvate metabolisms in nuclear and cytoplasmic maturation are still unclear, especially in chemically defined media.

In the present study, we examined the roles of glucose and pyruvate in a chemically defined medium on the morphological progress of meiosis and the cytoplasmic maturation, as determined by intracellular glutathione content and developmental competence, of porcine oocytes.

2. Materials and methods

2.1. Chemicals and Culture Media

Potassium chloride, KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical Co., Ltd (Osaka, Japan). Sodium chloride and paraffin liquid were obtained from Nacalai Teque Inc. (Kyoto, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan).

The medium used for collecting and washing oocyte-cumulus complexes (OCC) was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 μg gentamicin/mL and 65 μg potassium penicillin G/mL. The basic in vitro maturation (IVM) medium used was modified BSA-free North Carolina State University 37 medium [16] supplemented with 0.6 mM cysteine, 5 μg insulin/mL, 50 μM beta-mercaptoethanol and 0.2% (w/v) polyvinylalcohol (mNCSU37-PVA). This IVM medium supports blastocyst development and the birth of piglets following IVF and embryo transfer when porcine follicular fluid is used instead of 0.2% polyvinyl alcohol [17]. The medium for in vitro development to the blastocyst stage was mNCSU37 supplemented with 0.6 mM cysteine, 5 μg insulin/mL, 50 μM beta-mercaptoethanol and 0.4% (w/v) BSA (mNCSU37-BSA). In some experiments,the medium composition was modified as detailed below. All media (except modified TL-HEPES-PVA) were equilibrated under paraffin liquid at 39°C in an atmosphere of 5% CO_2 in air overnight prior to use.

2.2. Preparation and culture of cumulus-oocyte complexes

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 mg potassium penicillin G/L and 50 mg streptomycin sulphate/L. Using an 18-gauge needle and a disposable 10-mL syringe, OCC were aspirated from antral follicles (3 to 6 mm in diameter) on the surface of ovaries, and washed three times with modified TL-HEPES-PVA medium at room temperature (25 ^{o}C) [17]. Forty to fifty OCC with uniform ooplasm and a compact cumulus cell mass were washed three times with IVM medium. These complexes were subsequently cultured in 500 μL of IVM medium supplemented with

gonadotropins (10 iu eCG/mL and 10 iu hCG/mL) and 1mM dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP), for 20 h at 39°C in an atmosphere of 5% CO_2 in air. The OCC were washed three times in unsupplemented medium, transferred to 500 μL of fresh unsupplemented IVM medium and cultured for an additional 24 h [17,18]. Oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and evaluated for nuclear and cytoplasmic maturation. Oocytes were mounted, fixed for 48 h or more in 25% (v/v) acetic acid: alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and then examined under a phase-contrast microscope at 400x magnification. Nuclear morphology of oocytes at GV stages, and the meiotic stage of oocytes that had undergone GVBD, were classified into categories according to previous studies [17,19,20].

2.3. Assay of glutathione (GSH)

The intracellular content of glutathione (GSH) was measured as described previously [21]. Briefly, OCC were denuded by pipetting with 0.1% (w/v) hyaluronidase and washed three times in modified TL-HEPES-PVA medium and then another three times in stock buffer, 0.2M-sodium phosphate containing 10mM Na_4-EDTA (pH7.2). Five μL of buffer containing 30 mature oocytes was transferred to a 1.5-mL microtube and 5 μL of 1.25 M phosphoric acid was added; samples were frozen immediately (-80°C) and kept in the freezer until assayed. The GSH content of oocytes was determined by the DTNB-GSSG reductase recycling assay [22]. The total amount of GSH measured was divided by the number of oocytes in the sample to obtain the mean content per oocyte (pmol/oocyte).

2.4. Electrical activation and in vitro culture of oocytes

Electrical activation was carried out according to Miyoshi et al. [23] with modifications. The medium used for activation was 250.3 mM sorbitol, 0.3 mM HEPES, 0.2 mM hemi-calcium lactate and 0.2%(w/v) BSA. Denuded oocytes were washed once with activation medium and then placed between the two wire electrodes (1 mm apart) in activation medium; a direct-current pulse of 100 V/mm for 30 micro sec was applied twice at an interval of 1 min. Just after electrical stimulation, the oocytes were incubated in 500 μL of mNCSU37-BSA supplemented with 2.2 mg/mL

cytochalasin B for 2 h, and then cultured in cytochalasin B-free mNCSU37-BSA medium for 7 days. Cleavage and morula/blastocyst formation of the activated oocytes were examined on Days 2 and 7 of culture, respectively.

2.5. Experimental design

In the first experiment, to determine effect of glucose on resumption of meiosis in porcine oocytes, OCC were cultured in pyruvate-free IVM medium with or without 5.55 mM glucose as detailed above, then denuded and fixed to observe meiotic status.

In the second experiment, to determine whether glucose promoted resumption of meiosis in porcine oocytes though the PPP pathway, OCC were cultured in IVM medium with glucose plus varying concentrations of 6-aminonicotinamide (6-AN; 0, 10, 50 and 100 μM) or diphenyleneiodonium (DPI; 0, 10, 50 and 100 nM), inhibitors of the PPP. The meiotic status of the oocytes was examined at the end of IVM culture.

In the third experiment, the effect of various concentrations (0, 0.5, 2.5 and 5.0 mM) of sodium pyruvate in glucose-free IVM medium on resumption of meiosis in oocytes was evaluated. These concentrations were chosen from a similar range with physiological level of pyruvate (0.26 mM in human follicular fluid) and glucose concentration (5.55 mM) in IVM medium.

In the fourth experiment, the effects of glucose (5.55 mM) or pyruvate (5.55 mM) in the absence or presence of 10 μM 6-AN on oocyte glutathione content was determined. At the end of IVM culture, these oocytes were denuded and the intracellular GSH content was measured.

In the fifth experiment, the effects of 5.55 mM glucose and 5.55 mM sodium pyruvate, used both individually and in combination, on nuclear and cytoplasmic maturation of oocytes was evaluated. At the end of IVM culture, some of the oocytes were denuded and fixed to observe the meiotic status. Others were electrically activated, and the developmental competence of the oocytes was examined.

2.6. Statistical analysis.

All oocytes were randomly distributed within each experimental group and each experiment

was repeated at least 3 times. All percentage data were subjected to arc sine transformation before statistical analysis. Statistical analyses of results from 13-15 replicated samples in GSH assay and 3-7 replicates in others were used for treatment comparisons and carried out by analysis of variance (ANOVA) using the JMP 5.0 (SAS Institute, Inc., Cary, NC) program. If the P value was smaller than 0.05 in ANOVA, Tukey-Kramer's HSD test was followed using the same program. All data were expressed as means ± S.E.M. $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of glucose and gonadotropins on resumption of meiosis (Exp. 1)

As shown in Table 1, the proportions of oocytes undergoing GVBD and developing to the metaphase-II stage were significantly lower in IVM medium lacking glucose than in medium containing glucose: compared with control oocytes, significantly more of those cultured in glucose-free medium were arrested at the GV-I and GV-IV stages.

3.2. Effect of inhibitors of the PPP on resumption of meiosis (Exp. 2)

Supplementation of glucose-containing IVM medium with inhibitors of the PPP significantly inhibited GVBD in a concentration-dependent manner (Fig. 1); maximal inhibition was obtained using 50 and 100 μM of 6-AN and 50 and 100 nM of DPI. Most of the arrested oocytes were arrested at the GV-I stage (data not shown).

3.3. Effect of various concentrations of sodium pyruvate on resumption of meiosis and the progress (Exp. 3)

When OCC were cultured in glucose-free IVM medium containing various concentrations (0, 0.5, 2.5 and 5.0 mM) of sodium pyruvate, the percentage of oocytes undergoing GVBD and meiotic maturation increased at a higher concentration of pyruvate (Table 2). Supplementation with 5.0 mM pyruvate induced GVBD in 95.9% of the oocytes. Although ~50% of oocytes cultured in the presence of both 2.5 and 5.0 mM pyruvate were at metaphase-I, more of the oocytes cultured in 5.0 mM pyruvate reached the metaphase-II stage (Table 2).

3.4. Effect of glucose and pyruvate on oocyte glutathione content (Exp. 4)

The glutathione content of oocytes cultured in the presence of 5.55 mM glucose, without or with 10 μM 6-AN (3.22±0.35 and 2.96±0.57 pmol/oocyte, respectively) did not differ significantly from that of oocytes cultured in the absence of any energy substrates (2.95±0.37 pmol/oocyte; Fig. 2). However, glutathione content was significantly higher (5.60±0.97 pmol/oocyte) when 5.55 mM glucose was replaced with the same concentration of pyruvate; the inclusion of 10 μM 6-AN resulted in a much lower glutathione content (2.06±0.28 pmol/oocyte), which was equal to that in oocytes without any energy supplementation.

3.5. Effect of glucose and pyruvate on oocyte developmental competence (Exp. 5)

When oocyte maturation in the presence of 5.55 mM glucose only, 5.55 mM pyruvate only and the two in combination was compared, the incidences of GVBD and development to metaphase-I did not differ significantly (Table 3). However, there were significant differences in the proportions of oocytes reaching the metaphase-II stage. The lowest value (~23%) was observed in the pyruvate only group; significantly more had matured in the glucose only group (~51%) and more than 75% had matured when both pyruvate and glucose were present. The incidence of oocytes developing to or beyond the morula stage after parthenogenetic activation was higher in oocytes matured in the presence of both glucose and pyruvate, compared with control oocytes matured in the presence of glucose only (Table 4). However, the percentage of oocytes developing to the blastocyst stage did not differ.

4. Discussion

In the present study, the continuous presence of 5.55 mM glucose in a pyruvate-free chemically defined IVM medium was required to support GVBD and development to the metaphase-II stage in porcine oocytes. In the absence of glucose, oocyte progression beyond the GV-I stage occurred in only about half the oocytes, even if dibutyryl cAMP and gonadotropins were present during the first 20 h of culture for maturation, and essentially none reached metaphase-II. We then investigated the effects of 6-AN, an inhibitor of NADP-requiring enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) in the PPP, and DPI, a general inhibitor of NADPH oxidase. 6-aminonicotinamide is known to be an effective inhibitor of PPP activity in mouse oocytes [5] and bovine zygotes [24]. Diphenyleneiodonium has been shown to inhibit the flavoenzyme nitric oxide synthase (NOS) which generates nitric oxide in the presence of NADPH [25], and to inhibit reactive oxygen species (ROS) generation in mouse spermatozoa [26] and two-cell embryos [27]. In the present study, inclusion of these PPP inhibitors significantly inhibited GVBD: the majority of oocytes were arrested at GV-I, despite the presence of gonadotropins and dibutyryl cAMP during the first 20 h of IVM. In an earlier study, we demonstrated that supplementation of a glucose-containing medium with dibutyryl cAMP and gonadotropins for 20 h induces morphological progression of porcine oocytes from the GV-I to GV-II stage [17]. Therefore, glucose metabolism, especially NADPH production through the PPP or other pathways, appears to be closely associated with early changes in GV morphology that precede GVBD. A recent study has shown that supplementation of a chemically defined maturation medium with 100 nM DPI reduced the incidence of porcine oocytes at the metaphase-II stage after maturation culture for 40-44h, whereas a majority of the oocytes had undergone GVBD [14].

The current study demonstrated that replacement of glucose with pyruvate also supported resumption of meiosis in porcine oocytes, but only ~25% reached metaphase-II. Inclusion of both glucose and pyruvate significantly improved the proportion of oocytes that matured to metaphase-II, compared with either glucose or pyruvate used individually. This additive effect suggests that in addition to pyruvate production from glucose metabolism via glycolysis, metabolism of glucose through another metabolic pathway, such as the PPP, is required for full

stimulation of porcine oocytes maturation in mNCSU37. This supports a previous report by Downs and Hudson [11] that optimal meiotic maturation in mouse oocytes requires both pyruvate and glucose, although each used alone was able to support meiotic progression of ~45-65% of oocytes to the metaphase-II stage. However, it is still unclear the mechanism why the incidence of metaphase-I oocytes increased when sufficient concentration of pyruvate, which was more than 10-fold physiological, was supplemented glucose-free modified NCSU-37 medium (an amino acid-free chemically defined medium). Further experiments are required to clarify the phenomenon.

In porcine oocytes, a high level of intracellular GSH content, obtained by supplementing maturation medium with cysteine and beta-mercaptoethanol is known to be important promoting male pronuclear formation [28,29] and obtaining good embryonic development [17,30]. However, the effect of energy substrates on GSH content is unclear. In the current study, we found that the intracellular GSH content of porcine oocytes was significantly higher when the chemically defined maturation medium contained 5.55 mM pyruvate rather than glucose. This result supports recent observation that both cytosolic and mitochondrial metabolism of pyruvate regulates cytosolic NADPH levels, which may be a critical regulator of GSH production during maturation of mouse oocytes, via cytosolic NADP-dependent isocitrate dehydrogenase [31]. However, our results demonstrated that inclusion of 6-AN alone with pyruvate significantly reduced the GSH content of oocytes, to a level similar to that found in oocytes matured in medium containing glucose with/without 6-AN. This result suggests that the PPP may be associated with a supply of cytosolic NADPH in porcine oocytes. Furthermore, developmental competence to or beyond the morula stage was also increased when OCC were cultured in the presence of both glucose and pyruvate. These results suggest that an abundant supply of pyruvate, probably consumed for ATP production in the tricarboxylic acid cycle and NADPH production via cytosolic NADP-dependent isocitrate dehydrogenase and PPP, is important to synthesize GSH and to improve the developmental competence of oocytes in the pig. It has been reported that both oocyte glutathione content and developmental competence were changed and correlated when three types of maturation media, which contained glucose, were compared for porcine oocytes, whereas oocyte ATP contents did not differ among media [32]. There was also a correlation between

developmental competence and oocyte GSH content when the oocytes were protected from oxidative stress [33,34]. In a chemically defined medium, intracellular GSH content of porcine oocytes decreased when a PPP inhibitor was present, and further decreased when mitochondrial ATP production and the PPP were simultaneously inhibited [14]. One role of the PPP is to generate NADPH [35]. NADPH is known to be important both for the GSH reductase/peroxidase systems to protect cells against excessive ROS and for NADPH-oxidase to promote physiological levels of ROS involved in signaling pathway [36]. An abundant supply of pyruvate should be important to maintain a healthy state of porcine oocytes, including the level of intracellular glutathione.

In conclusion, results obtained in the present study demonstrate that glucose plays a critical role in resumption of meiosis in porcine oocytes, possibly through the PPP and cytosolic NADP-dependent isocitrate dehydrogenase for NADPH production, and that cytoplasmic maturation as assessed by glutathione content and developmental competence of oocytes to and beyond the morula stage, are improved probably through pathways associated with pyruvate. Based on the results, we would recommend adding Na-pyruvate to a glucose containing, chemically defined medium, such as mNCSU37 for IVM.

References

1. Downs SM, Utecht AM. Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. Biol Reprod 1999;60: 1446-1452.
2. Krisher RL, Bavister BD. Enhanced glycolysis after maturation of bovine oocytes in vitro is associated with increased developmental competence. Mol Reprod Devel 1999;53: 19-26.
3. O'Brien JK, Dwarte D, Ryan JP, Maxwell WMC, Evens G. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. Reprod Fertil Dev 1996;8: 1029-1037.
4. Downs SM, Humpherson PG, Martin KL, Leese HJ. Glucose utilization during gonadotropin-induced meiotic maturation in cumulus cell-enclosed mouse oocytes. Mol Reprod Devel 1996;44: 121-131.
5. Downs SM, Humpherson PG, Leese HJ. Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway. Biol Reprod 1998;58: 1084-1094.
6. Fagbohun CF, Downs SM. Matabolic coupling and ligand-stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. Biol Reprod 1991;45: 851-859.
7. Downs SM. A gap junction-mediated signal, rather than an external paracrine factor, predominates during meiotic induction in isolated mouse oocytes. Zygote 2001;9: 71-82.
8. Fagbohun CF, Downs SM. Requirement for glucose in ligand-stimulated meiotic maturation of cumulus cell-enclosed mouse oocytes. J Reprod Fertil 1992;96: 681-697.
9. Downs SM, Mastropolo AM. The participation of energy substrates in the control of meiotic maturation in murine oocytes. Dev Biol 1994;162: 154-168.
10. Downs SM. The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. Dev Biol 1995;167: 502-512.
11. Downs SM, Hudson ED. Energy substrates and the completion of spontaneous meiotic maturation. Zygote 2000;8: 339-351.
12. Zheng P, Wang H, Bavister BD, Ji W. Maturation of rhesus monkey oocytes in chemically defined culture media and their functional assessment by IVF and embryo development. Hum Reprod 2001;16: 300-305.
13. Steeves TE, Gardner DK. Metabolism of glucose, pyruvate, and glutamine during the maturation of oocytes derived from pre-pubertal and adult cows. Mol Reprod Devel 1999;54: 92-101.
14. Herrick JR, Brad AM, Krisher RL. Chemical manipulation of glucose metabolism in porcine oocytes: effects on nuclear and cytoplasmic maturation in vitro. Reproduction 2006;131: 289-298.
15. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003;126: 197-204.
16. Reed ML, Illera MJ, Petters RM. *In vitro* culture of pig embryos. Theriogenology 1992;37: 95-109.
17. Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following in vitro fertilization. Biol Reprod 1997;57: 49-53.
18. Funahashi H, Cantley TC, Day BN. Different hormonal requirement of porcine oocyte-complexes during maturation *in vitro*. J Reprod Fertil 1994;101: 159-165.
19. Motlik J, Fulka J. Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. J Exp Zool 1976;198: 155-162.
20. Hunter RHF, Polge C. Maturation of follicular oocytes in the pig after injection of human chorionic gonadotrophin. J Reprod Fertil 1966;12: 525-531.
21. Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN. Use of low salt culture medium for in vitro maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in vitro fertilization. Biol Reprod 1994;51: 633-639.
22. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. In: Meister A (ed), Methods in enzymology vol.113; Glutamate, glutamine, glutathione, and related compounds: Academic Press, 1985;548-555.
23. Miyoshi K, Yuki Y, Yoshida M. Optimization of Ca2+ concentrations in fusion and activation media for production of cloned embryos from miniature pig somatic cells. J

Reprod Dev 2005;51: 699-706.
24. Comizzoli P, Urner F, Sakkas D, Renard JP. Up-regulation of glucose metabolism during male pronucleus formation determines the early onset of the s phase in bovine zygotes. Biol Reprod 2003;68: 1934-1940.
25. Stuehr D, Fasehun O, Kwon N, Gross S, Gonzalez J, Levi R, Nathan C. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. FASEB J 1991;5: 98-103.
26. Ecroyd HW, Jones RC, Aitken RJ. Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation. Biol Reprod 2003;69: 347-354.
27. Nasr-Esfahani MH, Johnson MH. The origin of reactive oxygen species in mouse cultured in vitro. Development 1991;113: 551-560.
28. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993;49: 89-94.
29. Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN. Use of low-salt culture medium for in vitro maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in vitro fertilization. Biol Reprod 1994;51: 633-639.
30. Abeydeera LR, Wang WH, Cantley TC, Prather RS, Day BN. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology 1998;50: 747-756.
31. Dumollard R, Ward Z, Carroll J, Duchen MR, Dumollard R, Ward Z, Carroll J, Duchen MR. Regulation of redox metabolism in the mouse oocyte and embryo. Development 2007;134: 455-465.
32. Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, Clifford AL, Krisher RL. Glutathione and adenosine triphosphate content of in vivo and in vitro matured porcine oocytes. Mol Reprod Devel 2003;64: 492-498.
33. Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. Biol Reprod 2001;65: 1800-1806.
34. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T, Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 2004;71: 1150-1157.
35. Tian WN, Braunstein LD, Pang J, Stuhlmeier KM, Xi QC, Tian X, Stanton RC. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. J Biol Chem 1998;273: 10609-10617.
36. Forman HJ, Torres M. Redox signaling in macrophages. Molecular Aspects of Medicine 2001;22: 189-216.

Legend of figures

Fig. 1. Effect of various concentrations of 6-AN and DPI on meiotic resumption of porcine oocytes. Bars show the proportion of all oocytes at GVBD to metaphase-II. Numbers in parentheses indicate the total number of oocytes examined. Bars with different letters within the same PPP inhibitor differ significantly ($P < 0.05$).

Fig. 2. Glutathione content in porcine oocytes matured in the presence of either 5.55 mM glucose or 5.55 mM Na-pyruvate, with or without 10 μM 6-aminonicotinamide (6-AN). Numbers in parentheses indicate the total number of oocytes assayed. Bars with different letters differ significantly ($P < 0.05$).

Fig. 1.

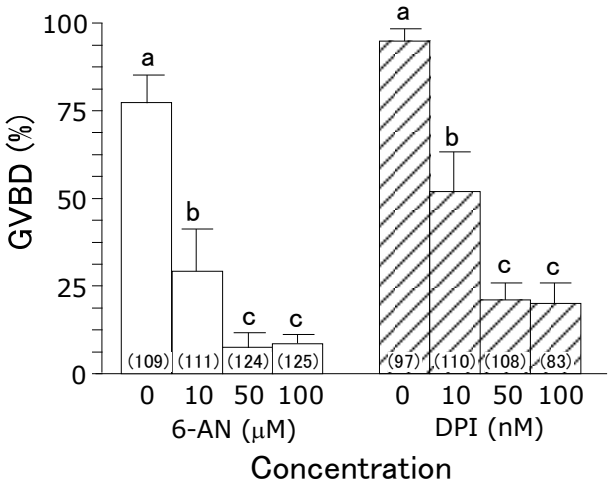
GVBD (%)
100
75
50
25
0
a
b
c
c
a
b
c
c
(109)
(111)
(124)
(125)
(97)
(110)
(108)
(83)
0
10
50
100
0
10
50
100
6-AN (μM)
DPI (nM)
Concentration

Fig. 2

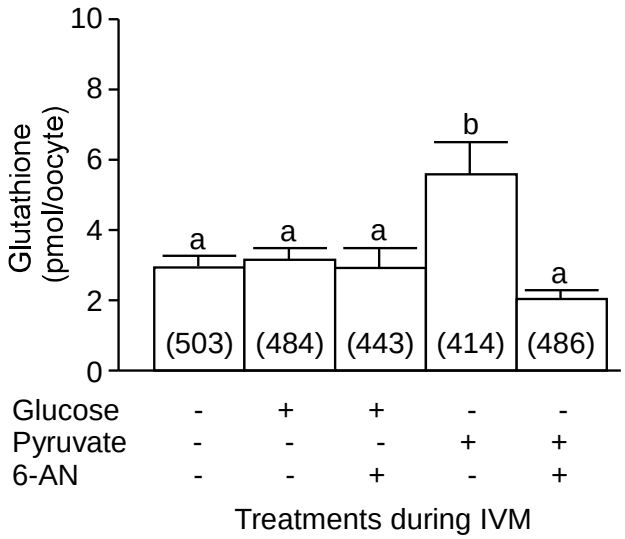
Glutathione (pmol/oocyte)
10
8
6
4
2
0
a
a
a
b
a
(503)
(484)
(443)
(414)
(486)
Glucose - + + - -
Pyruvate - - - + +
6-AN - - + - +
Treatments during IVM

Table 1. Effect of glucose on the resumption of meiosis in porcine oocytes

Concentration of Glucose (mM)	No. of oocytes examined*	%** oocytes at the stage of:				% oocytes completing GVBD	% M-II oocytes
		GV-I	GV-II	GV-III	GV-IV		
0	210	48.7 ± 8.6 [a]	8.2 ± 5.4	9.8 ± 4.5	12.0 ± 2.9 [a]	21.3 ± 4.4 [a]	0.7 ± 0.7 [a]
5.55	191	5.5 ± 3.1 [b]	3.2 ± 1.9	0.8 ± 0.5	1.8 ± 1.8 [b]	88.7 ± 3.6 [b]	57.6 ± 4.6 [b]

* Oocytes were cultured for 20 h in pyruvate-free mNCSU37-PVA containing 10 iu/mL eCG, 10 iu/mL hCG and 1 mM dibutyryl cAMP and then for 24 h without these supplements.

**Percentage based on the total number of oocytes examined.

Data are given as mean ± S.E.M. from five replicated experiments.

Values with different superscripts within columns are significantly different ($P < 0.05$).

Table 2. Effect of pyruvate in glucose-free maturation medium on the resumption of meiosis in porcine oocytes

Concentration of Na-pyruvate (mM)	No. of oocytes examined*	%** oocytes completing GVBD	%** M-I oocytes	%** M-II oocytes
0	230	10.4 ± 3.6[a]	4.0 ± 3.1[a]	0.5 ± 0.5[a]
0.5	226	41.5 ± 4.9[b]	8.3 ± 2.5[a]	7.6 ± 3.6[a]
2.5	221	92.5 ± 2.7[c]	51.6 ± 8.2[b]	18.2 ± 6.2[ab]
5.0	218	95.9 ± 1.1[c]	54.6 ± 9.0[b]	25.8 ± 5.0[b]

*Oocytes were cultured for 20 h in glucose-free mNCSU37-PVA containing 10 iu/mL eCG, 10 iu/mL hCG and 1 mM dibutyryl cAMP and then for 24 h without these supplements.

**Percentage based on the total number of oocytes examined

Data are given as mean ± S.E.M. from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).

Table 3. Effect of glucose and pyruvate on the resumption of meiosis in porcine oocytes

Energy substrates*	No. of oocytes Examined**	%*** oocytes completing GVBD	%*** M-I oocytes	%*** M-II oocytes
G	138	95.7 ± 3.3	43.5 ± 3.7[a]	50.6 ± 2.7[a]
P	146	92.2 ± 3.3	53.4 ±14.8[a]	23.3 ± 9.3[b]
G + P	156	93.4 ± 3.6	10.9 ±5.5[b]	76.3 ± 7.2[c]

*G; 5.55mM glucose, P; 5.55.mM Na-pyruvate.

** Oocytes were cultured for 20 h in mNCSU37-PVA containing 10 iu/mL eCG, 10 iu/mL hCG and 1 mM dibutyryl cAMP and then for 24 h without these supplements.

***Percentage based on the total number of oocytes examined.

Data are given as mean ± S.E.M. from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).

Table 4. Effect of pyruvate in the maturation medium on the development of electrically activated porcine oocytes in vitro

Presence of Na-pyruvate*	No. of oocytes activated**	%*** oocytes cleaved	%*** oocytes developing beyond the morula stage	%*** oocytes developing to the blastocyst
G	135	65.5 ±11.6	22.2 ± 5.2[a]	10.1 ± 4.2
G + P	146	81.2 ± 6.2	42.0 ± 4.0[b]	17.4 ± 3.1

*G; 5.55mM glucose, P; 5.55.mM Na-pyruvate.

**Oocytes were cultured for 20 h in mNCSU37-PVA containing 10 iu/mL eCG, 10 iu/mL hCG and 1 mM dibutyryl cAMP and then for 24 h without these supplements.

***Percentage based on the number of oocytes stimulated.

Data are given as mean ± S.E.M. from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).