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—Original Article—

Osmolarity- and Stage-Dependent Effects of Glycine on Parthenogenetic Development of Pig Oocytes

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Abstract. The osmolarities of media that are most effective for *in vitro* culture of mammalian oocytes and embryos are lower than that of oviductal fluid. Oocytes and embryos can survive the high physiological osmolarity *in vivo* perhaps owing to the presence of amino acids such as glycine, which serve as organic osmolytes in the female reproductive tract. In the present study, the effects of glycine on the parthenogenetic development of pig oocytes were examined in hypotonic or isotonic media. The results showed that culturing oocytes in isotonic media improved the cleavage rates ($P<0.01$) at 2 days in culture but inhibited any further development beyond cleavage when compared with the hypotonic media. However, addition of 4 mM glycine to the isotonic media resulted in improved blastocyst formation rates compared with that observed in the hypotonic media ($P<0.01$), and there was no inhibition of development beyond the cleavage stages in oocytes. The beneficial effects of glycine were observed only when oocytes were cultured in isotonic media and glycine was added at day 2 or 3 in culture. The results from the present study indicate that an isotonic medium with glycine is useful for *in vitro* culture of pig oocytes and that glycine may protect pig oocytes against the detrimental effects of increased osmolarity.
Key words: Glycine, *In vitro* development, Isotonic medium, Oocyte, Osmolarity

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Establishment of *in vitro* culture systems for supporting the early development of mammalian embryos is important in basic research for studying the mechanism controlling their development, as well as for applications such as somatic cell nuclear transfer (SCNT) and gene injection.

The osmolarity of the culture medium is an important factor for mammalian embryonic development. The osmolarity of mouse oviductal fluid was calculated to be above 340 mOsm [1, 2]. A similarly high osmolarity was predicted for human fallopian tube fluid based on electron probe measurements [3]. Direct measurements revealed the osmolarity of mouse oviductal fluid to be 290–300 mOsm [4]. Interestingly, even though the physiological osmolarity is very high, the most effective media for culturing mammalian embryos from the 1-cell to blastocyst stages have very low osmolarities, in the range of 240–280 mOsm [5–9]. Osmolarities greater than 300 mOsm are detrimental to mammalian embryos [2, 6, 7, 10, 11]. Porcine zygote medium-3 (PZM-3) [12] at 260–280 mOsm [13, 14] is the most common culture medium used in pigs for 1-cell embryos produced *in vitro* and *in vivo*, artificially activated oocytes and cloned embryos produced by nuclear transfer. The osmolarity of PZM-3 is much lower than the physiological osmolarity of 318 to 321 mOsm for pig oviductal fluid [13].

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The survival of embryos at a high physiological osmolarity *in vivo* appears to be due to amino acids such as glycine [15], glutamine [16] and β-alanine [17] that serve as organic osmolytes in the female reproductive tract. Recently, Li *et al.* [13] reported that the concentrations and composition of free amino acids in the female pig reproductive tract are very different from the formulation of PZM-3. At day 3 after the onset of estrus, the total concentration of free amino acids in oviductal fluid was found to be four times higher than that in PZM-3. Remarkably, the concentration of glycine at that stage was 41 times higher in the fluid than in PZM-3.

Therefore, inclusion of glycine in the culture medium at the concentrations present in pig oviductal fluid could potentially enable pig oocytes and embryos to develop *in vitro* at the physiological osmolarity. To test this hypothesis, the effects of glycine on the parthenogenetic development of pig oocytes were investigated in hypotonic or isotonic media *in vitro* in the present study.

Materials and Methods

In vitro maturation of oocytes

Pig ovaries were collected from prepubertal gilts at a local slaughterhouse and transported at 32–35 C in saline to the laboratory. The follicular contents were recovered by aspiration from the follicles (2–5 mm in diameter) using an 18-gauge needle (Terumo, Tokyo, Japan) and a 5-ml disposable syringe (Nipro, Osaka, Japan). The cumulus-oocyte complexes (COCs) were collected from the follicular contents and washed twice with a) HEPES (Nacalai Tesque, Kyoto, Japan)-buffered Tyrode solution supplemented with lactate, pyruvate and polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA) and b) maturation medium, respectively. The maturation

medium was composed of 90% (v/v) TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate (Sigma), 3.05 mM D-glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.57 mM cysteine hydrochloride hydrate (Sigma), 10 ng/ml epidermal growth factor (Sigma), 10 IU/ml eCG (Aska Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (Aska), 100 μg/ml amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (w/v) PVA and 10% (v/v) pig follicular fluid. Only those COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. COCs were transferred to droplets (200 μl) of maturation medium in groups of 40 to 50 under paraffin oil (Nacalai Tesque) in a 35-mm polystyrene dish (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and cultured at 38.5 C in an atmosphere of 5% CO_2 in air. After 40 to 42 h in culture, cumulus cells were removed by pipetting with 0.1% (w/v) hyaluronidase (Sigma). Oocytes with polar bodies were selected for further experiments.

Activation and culture of oocytes

In vitro-matured and denuded oocytes were washed twice in activation media composed of 250.3 mM sorbitol, 0.5 mM $Ca(CH_3COO)_2$, 0.5 mM $Mg(CH_3COO)_2$ and 0.1% (w/v) bovine serum albumin [18]. Then, the oocytes were transferred in groups of 30 to 60 to a well of a 4-well plate (Nunc, Roskilde, Denmark) containing 800 μl of the activation medium. The ultrasound probe (8 mm in diameter) of a KTAC-3000 Sonopore (Nepa Gene, Chiba, Japan) was inserted directly into the activation media, and the oocytes were exposed to 2872 kHz at 45 V for 30 sec, with a 10 Hz burst rate and a 30% duty cycle. A miniature stirrer was placed within the wells and stirred at 300 rpm during exposure to ultrasound. Modified PZM-3 (mPZM-3), in which 50 μg/ml gentamicin was replaced with 100 μg/ml amikacin sulfate [19], was used as the standard medium for oocyte culture. After ultrasound exposure, the oocytes were transferred to 50 μl of culture medium supplemented with 2.2 μg/ml cytochalasin B to prevent extrusion of a second polar body. After incubation for 2 h at 38.5 C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2, the oocytes were transferred into 50 μl of the same medium without cytochalasin B and cultured further. The oocytes were examined for cleavage at day 2 in culture. At day 7, oocytes with blastocoels were placed on slides with drops of mounting media that consisted of glycerol and phosphate-buffered saline (9:1) with 100 μg/ml Hoechst 33342 (Sigma). Coverslips were placed on the oocytes, and the edges were sealed with nail polish. The nuclei were counted under ultraviolet light, and oocytes with 32 or more cells were designated as blastocysts.

Experimental designs

In Experiment 1, the effects of glycine in hypotonic or isotonic media (osmolarity adjusted with NaCl) on the parthenogenetic development of oocytes were examined. Oocytes were cultured in mPZM-3 or mPZM-3 with 30 mM NaCl (mPZM-3 + NaCl). The osmolarities of mPZM-3 and mPZM-3 + NaCl were 273 mOsm and 326 mOsm, respectively, when measured using a vapor pressure osmometer (Wescor, Logan, UT, USA). These media were supplemented either with or without 4 mM glycine, since the concentration of glycine in pig oviductal fluid is 4.1 mM at day 3 after estrus [13], and mPZM-3 already contains 0.1 mM glycine.

In Experiment 2, the effects of glycine in hypotonic or isotonic media (osmolarity adjusted with sucrose) on the parthenogenetic development of oocytes were examined. Oocytes were cultured in either mPZM-3 or mPZM-3 with 50 mM sucrose (mPZM-3 + sucrose; 318 mOsm), both of which were supplemented with or without 4 mM glycine.

In Experiment 3, the effects of osmolarity on the parthenogenetic development of oocytes cultured in media supplemented with glycine were tested. Oocytes were cultured in mPZM-3 with 4 mM glycine and NaCl at varying concentrations (0, 15, 30 or 45 mM). The osmolarities of the media with 0, 15, 30 and 45 mM NaCl were 273, 299, 326 and 361 mOsm, respectively.

In Experiment 4, the effects of varying concentrations of glycine on the parthenogenetic development of oocytes in isotonic media were tested. Oocytes were cultured in mPZM-3 + NaCl supplemented with 0, 1, 2, 4, 6 or 8 mM glycine.

In Experiment 5, the effects of glycine added in different periods for varying times were investigated on the parthenogenetic development of oocytes in isotonic media. Oocytes were cultured in mPZM-3 + NaCl to which 4 mM glycine was added on different days. In a preliminary experiment, the rates of blastocyst formation of oocytes cultured with glycine during days 1–2 or 3–4 in culture were significantly higher than those cultured without glycine, although the addition of glycine during days 5–7 did not affect oocyte development. Therefore, glycine was only added on one day (day 1, 2, 3 or 4) during culture.

Statistical analysis

Experiments were replicated five or six times. All percentage data were subjected to arcsine transformation in each replicate. The transformed values and numbers of cells in blastocysts were analyzed by one-way or two-way ANOVA, followed by Fisher's protected least significant difference test. A probability of $P<0.05$ was considered statistically significant.

Results

Experiment 1

As shown in Table 1, the cleavage rates of oocytes cultured in mPZM-3 + NaCl (77.5–82.2%) were significantly ($P<0.01$) higher than those cultured in mPZM-3 (49.0–51.8%), regardless of the presence or absence of glycine. The addition of glycine did not affect the rates of blastocyst formation in oocytes cultured in mPZM-3 (13.4–15.9%). Interestingly, the blastocyst formation rate of oocytes cultured in mPZM-3 + NaCl (12.3%) did not significantly differ from those cultured in mPZM-3 with or without glycine (13.4–15.9%); however, the blastocyst formation rate of oocytes cultured in mPZM-3 + NaCl with glycine was significantly ($P<0.01$) higher (23.3%). The proportion of blastocysts formed at day 7 relative to the number of oocytes cleaved at day 2 was calculated. The results showed that the rate of blastocyst formation for oocytes cultured in mPZM-3 + NaCl (15.3%) was significantly (at least $P<0.05$) lower than for those cultured in mPZM-3 with or without glycine or mPZM-3 + NaCl with glycine (25.9–32.9%). The mean numbers of cells (41.3–49.9) of the blastocysts were not affected by the addition of either salt or glycine.

Table 1. Effects of glycine and NaCl (to adjust osmolarity) on the parthenogenetic development of pig oocytes in hypotonic or isotonic media[a]

Medium	Glycine	No. of oocytes cultured	No. (mean % ± SEM)[b] of oocytes developed to		Mean % ± SEM of blastocysts per oocytes cleaved	Mean no. ± SEM of cells in blastocysts
			≥ 2-cell (Day 2)[c]	Blastocyst (Day 7)[c]		
mPZM-3	–	119	58 (49.0 ± 2.6)[d]	19 (15.9 ± 1.7)[d]	32.9 ± 3.8[d]	42.1 ± 2.0
	+	116	60 (51.8 ± 1.7)[d]	15 (13.4 ± 2.6)[d]	25.9 ± 4.9[d]	49.9 ± 4.0
mPZM-3 + NaCl	–	120	98 (82.2 ± 4.8)[e]	15 (12.3 ± 1.3)[d]	15.3 ± 2.0[e]	41.3 ± 1.9
	+	117	90 (77.5 ± 4.5)[e]	27 (23.3 ± 0.6)[e]	30.5 ± 1.8[d]	43.9 ± 2.5

[a] Oocytes were cultured in mPZM-3 (273 mOsm) or mPZM-3 with 30 mM NaCl (mPZM-3 + NaCl; 326 mOsm) supplemented with or without 4 mM glycine. [b] Percentage per oocytes cultured. [c] Days in culture. [d–e] Values with different superscripts within each column are significantly different (at least $P<0.05$).

Table 2. Effects of glycine and sucrose (to adjust osmolarity) on the parthenogenetic development of pig oocytes in hypotonic or isotonic media[a]

Medium	Glycine	No. of oocytes cultured	No. (mean % ± SEM)[b] of oocytes developed to		Mean % ± SEM of blastocysts per oocytes cleaved	Mean no. ± SEM of cells in blastocysts
			≥ 2-cell (Day 2)[c]	Blastocyst (Day 7)[c]		
mPZM-3	–	121	52 (42.6 ± 1.1)[d]	16 (13.1 ± 1.9)[d]	30.6 ± 4.2[d]	47.8 ± 3.9
	+	121	51 (41.4 ± 4.4)[d]	11 (8.8 ± 2.0)[d,e]	20.8 ± 3.8[e]	43.6 ± 4.0
mPZM-3 + sucrose	–	122	84 (68.9 ± 2.2)[e]	9 (7.4 ± 1.4)[e]	10.8 ± 2.1[f]	43.2 ± 4.1
	+	121	81 (66.9 ± 1.1)[e]	24 (19.9 ± 0.8)[f]	29.8 ± 1.4[d,e]	45.8 ± 3.1

[a] Oocytes were cultured in mPZM-3 (273 mOsm) or mPZM-3 with 50 mM sucrose (mPZM-3 + sucrose; 318 mOsm) supplemented with or without 4 mM glycine. [b] Percentage per oocytes cultured. [c] Days in culture. [d–f] Values with different superscripts within each column are significantly different (at least $P<0.05$).

Table 3. Effects of osmolarity on the parthenogenetic development of pig oocytes in media supplemented with glycine[a]

Osmolarity (mOsm)	No. of oocytes cultured	No. (mean % ± SEM)[b] of oocytes developed to		Mean no. ± SEM of cells in blastocysts
		≥ 2-cell (Day 2)[c]	Blastocyst (Day 7)[c]	
273	108	52 (48.1 ± 1.7)[d]	13 (12.0 ± 1.8)[d,e]	46.9 ± 4.5
299	109	64 (58.3 ± 2.5)[d]	19 (17.3 ± 3.8)[e,f]	45.8 ± 2.9
326	108	81 (74.9 ± 4.7)[e]	25 (23.1 ± 2.2)[f]	43.5 ± 1.7
361	109	92 (85.0 ± 4.0)[e]	8 (7.4 ± 1.9)[d]	35.8 ± 1.3

[a] Oocytes were cultured in mPZM-3 with 4 mM glycine and different concentrations of NaCl (0, 15, 30 or 45 mM). [b] Percentage per oocytes cultured. [c] Days in culture. [d–f] Values with different superscripts within each column are significantly different (at least $P<0.05$).

Experiment 2

As shown in Table 2, the cleavage rates of oocytes cultured in mPZM-3 + sucrose (66.9–68.9%) were significantly ($P<0.01$) higher than those cultured in mPZM-3 (41.4–42.6%), regardless of the presence or absence of glycine. The addition of glycine did not affect the blastocyst formation rates of oocytes cultured in mPZM-3 (8.8–13.1%). However, the blastocyst formation rates of oocytes cultured in mPZM-3 + sucrose were significantly increased ($P<0.01$) by the addition of glycine (19.9%). The proportion of blastocysts formed at day 7 relative to the number of oocytes cleaved at day 2 was calculated. The results showed that the blastocyst formation rate of oocytes cultured in mPZM-3 + sucrose (10.8%) was significantly ($P<0.01$) lower than that of those cultured in mPZM-3 (30.6%), although there were no significant differences between these two groups when glycine was added to both media (29.8–30.6%). The mean numbers of cells (43.2–47.8) in the blastocysts were not affected by the addition of either sucrose or glycine.

Experiment 3

Table 3 shows that the cleavage rates of oocytes cultured at 326 mOsm and 361 mOsm (74.9–85.0%) were significantly (at least $P<0.05$) higher than those cultured at 273 mOsm and 299 mOsm (48.1–58.3%). The blastocyst formation rate of oocytes cultured at 326 mOsm (23.1%) was significantly ($P<0.01$) higher than that of those cultured at 273 mOsm or 361 mOsm (7.4–12.0%). The mean numbers of cells (35.8–46.9) in the blastocysts were not affected by the changes in osmolarities.

Experiment 4

As shown in Table 4, the cleavage rates of oocytes were not affected by the different concentrations of glycine in media (71.6–80.4%). However, the blastocyst formation rate of oocytes cultured with 4 mM glycine in the medium (33.6%) was significantly ($P<0.01$) higher than those of the other groups (13.2–20.6%). The mean numbers of cells (42.3–47.5) in the blastocysts were not affected by the different

Table 4. Parthenogenetic development of pig oocytes in isotonic media supplemented with different concentrations of glycine[a]

Concentration of glycine (mM)	No. of oocytes cultured	No. (mean % ± SEM)[b] of oocytes developed to		Mean no. ± SEM of cells in blastocysts
		≥ 2-cell (Day 2)[c]	Blastocyst (Day 7)[c]	
0	101	81 (80.4 ± 2.4)	16 (16.1 ± 1.8)[d]	46.3 ± 2.9
1	98	76 (76.4 ± 4.1)	20 (20.6 ± 4.3)[d]	42.3 ± 2.1
2	100	77 (77.8 ± 2.8)	14 (13.2 ± 1.8)[d]	44.1 ± 2.6
4	98	72 (74.0 ± 2.4)	32 (33.6 ± 3.7)[e]	47.5 ± 2.4
6	99	70 (71.6 ± 3.0)	14 (13.6 ± 2.0)[d]	47.2 ± 3.7
8	99	72 (73.0 ± 2.9)	15 (15.4 ± 1.6)[d]	43.3 ± 3.4

[a] Oocytes were cultured in mPZM-3 with 30 mM NaCl (326 mOsm). [b] Percentage per oocytes cultured. [c] Days in culture. [d–e] Values with different superscripts are significantly different (P<0.01).

Table 5. Parthenogenetic development of pig oocytes in isotonic media supplemented with glycine on days 1, 2, 3, 4 or 1–7 in culture[a]

Glycine addition (days in culture)	No. of oocytes cultured	No. (mean % ± SEM)[b] of oocytes developed to		Mean no. ± SEM of cells in blastocysts
		≥ 2-cell (Day 2)[c]	Blastocyst (Day 7)[c]	
None	112	83 (73.6 ± 2.2)	15 (13.5 ± 1.1)[d]	46.1 ± 3.4
1	109	73 (66.9 ± 4.3)	20 (19.2 ± 2.8)[d,e]	46.9 ± 2.7
2	110	75 (68.5 ± 1.7)	25 (23.1 ± 1.5)[e,f]	47.5 ± 2.9
3	111	77 (69.9 ± 3.5)	28 (25.1 ± 1.9)[f]	50.2 ± 2.2
4	111	76 (68.0 ± 2.8)	17 (15.1 ± 2.7)[d]	52.9 ± 4.5
1–7	110	72 (65.8 ± 1.8)	25 (22.9 ± 1.4)[e,f]	46.6 ± 2.9

[a] Oocytes were cultured in mPZM-3 with 30 mM NaCl (326 mOsm) supplemented with 4 mM glycine in different periods during culture. [b] Percentage per oocytes cultured. [c] Days in culture. [d–f] Values with different superscripts are significantly different (at least P<0.05).

glycine concentrations.

Experiment 5

The results in Table 5 show that the cleavage rates of oocytes cultured in media with glycine added at days 1, 2, 3 or 4 in culture (66.9–69.9%) did not significantly differ from those of oocytes continuously cultured with or without glycine in the media (65.8–73.6%). However, the blastocyst formation rates of oocytes cultured in media with glycine added during day 2 or 3 of culture (23.1–25.1%) were significantly (P<0.01) higher than those cultured without glycine in the media (13.5%) and did not significantly differ from that of oocytes cultured with continuous glycine in the media (22.9%). In contrast, the blastocyst formation rates of oocytes cultured with glycine in the media during day 1 or 4 in culture (15.1–19.2%) did not significantly differ from those cultured without glycine in the medium (13.5%). The mean numbers of cells (46.1–52.9) in the blastocysts were not affected by the different time periods of glycine addition to the media.

Discussion

The results of the present study indicate that culture of artificially activated pig oocytes in isotonic media improves cleavage rates but inhibits development beyond the cleavage stages as compared with hypotonic media. Addition of glycine to the isotonic media prevents this inhibition of oocyte development, resulting in improved blastocyst formation rates compared with hypotonic media.

The results of the present study also show that the NaCl-induced changes in osmolarity from 273 mOsm to 326 mOsm improved the cleavage rates of pig oocytes at day 2 in culture. In contrast, development of oocytes beyond the cleavage stages was inhibited at 326 mOsm compared with 273 mOsm. Similar results were obtained when the osmolarity was changed from 273 mOsm to 318 mOsm by the addition of sucrose. Therefore, it is the change in osmolarity and not NaCl or sucrose specifically that is involved in the development of pig oocytes. These results demonstrate that the optimal osmolarities required before and after early cleavage for the parthenogenetic development of pig oocytes are different. Recent reports have indicated that there is a preference for physiological osmolarities during the first 2 to 3 days of culturing pig *in vitro*-fertilized embryos [13], activated oocytes [20–22] and SCNT embryos [14, 21, 22], although the oocytes/embryos have to be transferred into hypotonic media subsequently in order to maintain *in vitro* development. The results of the present study are consistent with these reports.

The main discovery from the present study is that glycine can support the parthenogenetic development of pig oocytes in isotonic media. Mammalian cells regulate their volumes osmotically by importing and exporting intracellular osmolytes to adjust the intracellular osmotic pressure and to maintain size. The first line of defense for

cells against an unwanted decrease in volume is to import inorganic ions [23]. However, in cells that are exposed to chronic hypertonicity, accumulation of a large number of ions is needed for sustaining cell size, which increases intracellular ionic strength but is detrimental to cell viability. In such cases, cells also accumulate small, uncharged compounds termed organic osmolytes, which replace a portion of the intracellular ions, thus reducing the ionic strength while still maintaining the cell volume [24, 25]. In mice, glycine accumulates to high intracellular levels and seems to function as an organic osmolyte during the earliest stages of embryonic development up to the 2-cell stage [2, 26, 27]. Steeves *et al*. [28] demonstrated that early mouse embryos use a mechanism that employs GLYT1 (a neurotransmitter transporter family member) for osmotically regulating the accumulation of glycine. In that study, it was shown that GLYT1 was required for embryonic viability and maintenance of normal cell volumes in oviductal fluid. The results of the present study show that the effects of glycine on oocyte development depend on the osmolarity of the media. Development to the blastocyst stage was enhanced in isotonic media but not in hypotonic media, suggesting that glycine functions as an organic osmolyte to protect pig oocytes against the detrimental effects of increased osmolarity. On the other hand, uptake of glycine in mouse oocytes and embryos by GLYT1 is Na^+ and Cl^- dependent. The reliance of this transport system on the inwardly directed Na^+ and Cl^- gradients allows highly concentration-dependent uptake of glycine and the maintenance of steep concentration gradients across the cell membrane, permitting a high glycine content in oocytes and embryos, even when the extracellular concentration is much lower [29]. Therefore, it is possible that the concentration of NaCl is also an important factor for oocyte development in the present study. Since GLYT1 activity is also found in early human embryos [29], the mechanism for glycine-dependent cell volume regulation could be common to mammals. Additional investigation is needed to examine the roles of GLYT1 in pig oocytes and embryos. The results from the present study provide insight into a novel *in vitro* culture system that could be used for the development of pig activated oocytes to the blastocyst stages at physiological osmolarity. When cultured in isotonic media during the first 2 days, the development of pig electrically activated oocytes and SCNT embryos improved because of the reduction in their fragmentation rates [21]. The same culture conditions also reduced apoptosis in pig SCNT embryos, resulting in improved development [14]. These results suggest that culture in hypotonic media induces excessive apoptosis and fragmentation in pig oocytes and embryos compared with isotonic media because of unphysiological conditions. Such apoptosis and fragmentation could possibly be prevented, resulting in effective production of blastocysts, as pig oocytes could be continuously cultured in media possessing physiological osmolarity by addition of glycine in the present study. We expect that this culture system could potentially be useful for nonsurgical transfer of *in vitro*-produced embryos into recipient females at the morula and blastocyst stages [30].

In the present study, the blastocyst formation rate of pig oocytes was at a maximum when 4 mM glycine was added to the culture media and the osmolarity was adjusted to 326 mOsm, i.e., under physiological conditions that occur in pig oviductal fluid. The final concentration of glycine was 4.1 mM because the medium originally contained 0.1 mM glycine. In contrast, when pig *in vitro*-fertilized embryos were cultured in modified PZM-3 (the concentrations and composition of amino acids were modified to mimic those in pig oviductal fluid at 3 days after estrus), addition of 4.1 mM glycine did not make a significant difference in blastocyst formation rates between 250 mOsm and 330 mOsm [13]. This contradiction in the results may be attributed to the effects of amino acids (other than glycine), which might have a negative effect on the development of pig embryos when their concentrations are modified.

The results of the present study also indicated that the effects of glycine on oocyte development vary according to the developmental stage of the oocytes. Development to the blastocyst stage in the isotonic medium was enhanced only when glycine was added during days 2 to 3 of culture, i.e., the 2-cell to 8-cell stages. The net rate of glycine appearance is high in pig oocytes that are *in vitro* matured in TCM-199 and *in vitro* fertilized, but it reaches a nadir at the 2-cell stage after culture for one day [31]. Therefore, we concluded that addition of glycine is not needed during day 1 of culture because pig oocytes contain an adequate amount of glycine at that time for protection against the detrimental effects of increased osmolarity. However, the glycine in pig oocytes is depleted by the 2-cell stage; therefore, it must be added during days 2–3 in culture when the oocytes are not transferred into a hypotonic medium. Even if glycine is added by days 4 to 7 of culture, accumulation of glycine might be too late to effectively replace inorganic ions in pig oocytes.

In conclusion, we established an effective *in vitro* culture system to support the parthenogenetic development of pig oocytes. This system would be useful in basic research for elucidating the mechanism controlling the development of oocytes. Further investigation is needed to address whether this system would be effective for culturing pig *in vitro*- or *in vivo*-fertilized embryos and SCNT embryos, as per our hypothesis.

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