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Short Communications

Replacement of glutamine with the dipeptide derivative alanyl-glutamine enhances *in vitro* maturation of porcine oocytes and development of embryos

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Summary

The presence of glutamine (Gln) in *in vitro* maturation (IVM) and *in vitro* culture (IVC) medium is a more potent factor for improving porcine oocyte and embryo development than other amino acids. However Gln is inherently unstable and spontaneously breaks down into ammonia, and therefore interferes with proper development. To avoid this adverse effect, Gln was replaced in the present study with its stable dipeptide derivative alanyl-glutamine (Ala-Gln) and the effects of this replacement on porcine IVM and IVC were evaluated. Replacement of Gln with Ala-Gln during IVM did not improve nuclear maturation, however numbers of early cleaved embryos were significantly increased after activation. Blastocyst formation rates were also significantly improved by using Ala-Gln during IVM. Replacement of Gln with Ala-Gln during IVC significantly increased total cell numbers in blastocysts. Blastocyst formation rate was also significantly higher when Ala-Gln was used in both IVM and IVC. In conclusion, the use of Ala-Gln rather than Gln gives better results for development in both porcine IVM and IVC.

Keywords: Alanyl-glutamine, GlutaMAX, Glutamine, *In vitro* maturation, Porcine embryo culture

Introduction

Glutamine (Gln) is the most abundant amino acid in porcine follicular fluid (Hong & Lee, 2007) and is constantly used as an energy substrate throughout development (Swain *et al.*, 2002). Similarly, addition of Gln to culture media improves *in vitro* development of porcine embryos (Petters *et al.*, 1990; Suzuki *et al.*, 2007). However, addition of Gln has a potential drawback due to its inherent instability as Gln spontaneously degrades into ammonia and 2-pyrrolidone-5-carboxylic acid (Vickery *et al.*, 1935). In the female reproductive tract, ammonia products are absorbed or neutralized by surrounding epithelial tissues, however in the case of *in vitro* culture they accumulate in the culture medium (Gardner & Lane, 1993). Accumulated ammonia is detrimental to mammalian oocytes and to embryo development (Hammon *et al.*, 2000).

The adverse effect of Gln can be avoided by using stable dipeptide derivatives such as alanyl-glutamine (Ala-Gln) or glycyl-glutamine (Roth *et al.*, 1988). Replacement of Gln with the dipeptide derivatives reduced the rates of polyspermy and apoptosis and increased cell numbers in blastocysts and the pregnancy rates in mice (Biggers *et al.*, 2004; Summers *et al.*, 2005). However, information on the effects of the dipeptide derivatives on porcine oocytes and/or embryo culture is scarce. Therefore, in the present study, we compared the use of Ala-Gln and Gln during *in vitro* maturation (IVM) and/or *in vitro* culture

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(IVC) of porcine oocytes/embryos in order to optimize porcine *in vitro* production (IVP) protocols.

Materials and methods

Materials

All chemicals and media were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) except for Ala-Gln, which was obtained from Invitrogen (GlutaMAX^{TM}-I; Carlsbad, CA, USA).

In vitro maturation of porcine oocytes

Porcine oocytes were matured *in vitro* following our standard protocol described elsewhere (Park *et al.*, 2010) but with slight modification. Briefly, cumulus–oocyte complexes (COCs) were aspirated from abattoir-derived porcine ovaries, washed three times and cultured in IVM medium supplemented with 0.5 μg/ml follicle-stimulating hormone and 0.5 μg/ml luteinizing hormone at 39°C, in 5% CO_2 in air for 22 h. The cultured COCs were washed and transferred to fresh IVM medium without hormones and cultured again for another 22 h under the same conditions. The IVM medium used was Medium 199 (without L-glutamine) supplemented with 10 ng/ml epidermal growth factor, 0.57 mM cysteine and 10% (v/v) porcine follicular fluid. For the experiments, either 2 mM L-Gln or Ala-Gln was added to the IVM medium.

Assessment of nuclear maturation of oocytes

Nuclear maturation was evaluated by presence of the first polar body. After IVM culture, oocytes were transferred to 0.1% hyaluronidase in IVM medium then pipetted gently until the cumulus cells were completely removed. Denuded oocytes were stained with 5 μg/ml of Hoechst 33342 for 20 min and observed under ultraviolet light.

Parthenogenetic activation and *in vitro* culture

Denuded oocytes were equilibrated in 280 mM mannitol solution supplemented with 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 0.01% PVA for 1 min, then transferred to a chamber between two electrodes 3.2 mm apart in the same mannitol solution. The oocytes were activated with a single direct current pulse of 1.5 kV/cm for 60 μs using an electrocell manipulator (BTX ECM 2001, Harvard Apparatus Inc., Holliston, MA, USA). Activated oocytes were cultured with Porcine Zygote Medium-3 (PZM-3) (Yoshioka *et al.*, 2002) under mineral oil at 39°C in a mixture of 5% CO_2, 5% O_2 and 90% N_2. Embryos were evaluated for cleavage on day 2 and for blastocyst formation and total cell numbers of blastocysts on day 7 of culture. In this study, embryos greater than 8-cell in size on day 2 were regarded as early cleavage stages. During IVC, 2 mM of L-Gln or Ala-Gln was added to PZM-3 as per the experimental design described below.

Counting blastocyst cell numbers

Blastocysts total cell numbers were counted after staining with 25 μg/ml Hoechst 33258 for 20 min. Stained blastocysts were mounted on a glass slide in a drop of glycerol, gently flattened with a cover glass and visualized under a fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis

All experiments were replicated at least five times and analysed statistically using Prism software (GraphPad, La Jolla, CA, USA). Student's *t*-test was used to analyze differences in maturation and early cleavage rates after IVM. One-way analysis of variance (ANOVA) was used to analyse cleavage and blastocyst rates and total cell number of blastocyst after IVC. A *P*-value less than 0.05 was considered to be significant.

Experimental design

For evaluating effects of Ala-Gln on IVM, oocytes were divided into two groups and cultured with Ala-Gln or Gln, respectively. After IVM, each group was activated and then cultured with PZM-3 with Gln for cleavage timing assessment.

For evaluating effects of Ala-Gln on IVC, oocytes were divided into four groups. Group G-g was cultured with Gln during both IVM and IVC. Group G-x was cultured with Gln during IVM and Ala-Gln for IVC. Group X-g was cultured with Ala-Gln for IVM and Gln for IVC. Group X-x was cultured with Ala-Gln during both IVM and IVC.

Results and Discussion

In the present study, we found that replacement of Gln with Ala-Gln improved both IVM and IVC results with culture of porcine oocytes/embryos. Oocyte quality after IVM culture can be assessed from two points of view, nuclear maturation and cytoplasmic maturation. Nuclear maturation involves resumption and progression of meiosis I and is assessed by extrusion of the first polar body. In this study, nuclear maturation was similar between Gln- and Ala-Gln-treated groups (Table 1). Cytoplasmic maturation involves structural redistribution of cellular organelles in the oocytes and synthesis of mRNA to initiate embryo

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Table 1. Effect of glutamine or alanyl-glutamine on *in vitro* nuclear maturation of porcine oocytes

	Oocytes examined (*n*)	Nuclear maturation (%)
Glutamine	329	295 (89.5 ± 0.5)
Alanyl-glutamine	332	300 (90.0 ± 1.3)

development. It is difficult to evaluate cytoplasmic maturation, however *in vitro* development after activation indirectly reveals the extent of cytoplasmic maturation of oocytes. In the present study, while there was no difference between the Gln- and the Ala-Gln-treated groups in the proportion of activated oocytes that cleaved at least once, the Ala-Gln group showed a significantly higher proportion of early cleavage stage embryos compared with the Gln group on day 2 after activation (Table 2; 33.0 ± 4.8% versus 16.7 ± 2.8%, respectively). It has been proven that early cleaved embryos in many mammalian species including pigs have more developmental competence (Isom *et al.*, 2011). We also observed in a previous study that early cleaved porcine embryos showed higher implantation rates (Koo *et al.*, 2010). Consistent with this finding, in the present study the blastocyst rate in the Ala-Gln-treated IVM group (X-g group in Table 3; 36.4 ± 3.6%) was significantly higher than in the Gln-treated group (G-g group in Table 3; 23.2 ± 2.7%).

Effects of Ala-Gln on IVC of parthenogenetic embryos were also assessed. Cleavage rates were similar among all the groups in this study (Table 3). However, blastocyst rates showed significant differences. As mentioned above, replacement of Gln with Ala-Gln during IVM led to a significantly improved blastocyst rate (between G-g and X-g groups). This significant improvement was also found between groups G-x (34.2 ± 3.8%) and X-x (51.3 ± 1.94%). In particular, the blastocyst rate in the X-x group was significantly higher compared with all the other groups.

On the other hand, total cell numbers of blastocysts were independent from IVM conditions: there were no significant differences between the G-g and X-g or between G-x and X-x groups. However, total blastocyst cell numbers were increased significantly in the G-x and X-x groups (63.5 ± 4.5 and 63.2 ± 2.7, respectively) compared with the G-g and X-g groups (41.9 ± 1.8 and 47.4 ± 1.6, respectively). This situation means that higher cell number in blastocysts is connected with IVC conditions. Previous reports have shown that embryo cell number is correlated with cell death, particularly apoptosis (Hardy, 1997; Yu *et al.*, 2007). The concentration of ammonia gradually increased during culture of embryos in the presence of Gln (Gardner & Lane, 1993). Ammonia induces apoptosis of blastomeres in a dose-dependent manner (Lane & Gardner, 2003). Consequently, reduced cell number in G-g and X-g groups in this study seems to be affected by accumulated ammonia from degraded Gln. As high cell number in blastocysts is critical for postimplantation development (Brison & Schultz, 1997), use of Ala-Gln rather than Gln to

Table 2. Effect of glutamine or alanyl-glutamine treatment during *in vitro* maturation on cleavage timing of porcine parthenogenetic embryos

	Embryos examined (*n*)	Cleaved embryos *n*(%)	2–4 cell stages *n*(%)	> 8-cell stage *n*(%)
Glutamine	279	233 (83.6 ± 2.9)	192 (83.3 ± 2.8)[a]	41 (16.7 ± 2.8)[a]
Alanyl-glutamine	281	248 (88.8 ± 1.7)	171 (67.0 ± 4.8)[b]	77 (33.0 ± 4.8)[b]

[a,b]Different superscripts within same column represent significant differences.

Table 3. Effect of glutamine or alanyl-glutamine treatment during *in vitro* maturation and/or *in vitro* culture on development of porcine parthenogenetic embryos

	Embryos examined (*n*)	Cleaved embryos *n*(%)	Blastocysts *n*(%)	Total cell number of blastocyts
G-g	153	136 (88.2 ± 2.0)	36 (23.2 ± 2.7)[a]	41.9 ± 1.8[a]
G-x	152	140 (91.4 ± 2.3)	52 (34.2 ± 3.8)[a,b]	63.5 ± 4.5[b]
X-g	222	200 (89.9 ± 2.1)	81 (36.4 ± 3.6)[b]	47.4 ± 1.6[a]
X-x	221	204 (92.0 ± 1.9)	113 (51.3 ± 1.9)[c]	63.2 ± 2.7[b]

[a–c]Different superscripts within same column represent significant differences. G-g: Gln on both IVM and IVC; G-x: Gln on IVM and Ala-Gln on IVC; X-g: Ala-Gln on IVM and Gln on IVC; X-x: Ala-Gln on both IVM and IVC.

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increase cell numbers of blastocyst is recommended for optimization of porcine IVP systems.

In conclusion, replacement of Gln with Ala-Gln during IVM did not improve nuclear maturation but did improve developmental competence after oocyte activation. Also, use of Ala-Gln during IVC increased the total cell number of blastocysts without reducing blastocyst formation rate. We therefore recommend the use of Ala-Gln in both IVM and IVC of porcine oocytes and embryos.

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