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Effect of Monosodium Glutamate on *In Vitro* Oocyte Maturation and Embryonic Development after Parthenogenesis in Pigs

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ABSTRACT

This study was designed to determine the effect of monosodium glutamate (MSG) on *in vitro* maturation (IVM) of oocytes and early development of parthenogenesis (PA) embryos in pigs. Each IVM and IVC medium was supplemented with various concentrations (0, 0.1, 0.5 and 5 mM) of MSG and non-essential amino acids (NEAA) depending on the experimental design. Immature pig oocytes were matured for 44 h and then oocytes reached metaphase II (MII) stage were electrically activated to induce parthenogenesis (PA). When immature oocytes were treated with MSG in the absence of NEAA during IVM, nuclear maturation (83.1-87.1%), intra-oocyte glutathione content, cumulus expansion, and cleavage (91.4-93.4%) of PA embryos were not influenced by MSG treatment at all concentrations. However, blastocyst formation of PA embryos was significantly increased by 5.0 mM MSG (45.3 ± 6.2%) compared to control (25.6 ± 3.4%). MSG treatment during IVM in the presence of NEAA did not show significant effect on nuclear maturation of oocytes and blastocyst formation after PA while 0.5 mM MSG (89.3 ± 1.9%) decreased ($P < 0.05$) cleavage of PA embryos compared to 0.1 mM MSG (94.6 ± 1.1%). When PA embryos were treated for 7 days with MSG during IVC, 5.0 mM MSG significantly decreased blastocyst formation (27.8 ± 4.9%) compared to no treatment (41.4 ± 1.9%) while no decrease in blastocyst formation was observed in 0.1 and 0.5 mM (37.4 ± 3.4% and 34.4 ± 2.6%, respectively). Our results demonstrated that 5 mM MSG in a NEAA-free chemically defined maturation medium showed positive effect on PA embryonic development while 5 mM MSG treatment during IVC was deleterious to PA embryonic development in pigs.

(Key Words : Monosodium glutamate, Oocyte maturation, Embryonic development, Parthenogenesis, Pig

INTRODUCTION

Recent progress in *in vitro* maturation of pig oocytes makes it possible to produce piglets by transferring *in vitro*-produced (IVP) pig embryos derived from IVM oocytes. However, despite of successful production of piglets, the developmental competence of IVP embryos derived from IVM oocytes is still inferior to their *in vivo*-developed counterparts which is mainly attributed to the lower quality of IVM oocytes compared to *in vivo* ones (Dobrinsky *et al*. 1996; Kang *et al.* 2009). For this reason, a wide variety of studies have been attempted to improve and optimize IVM system by determining effect of various factors such as energy substrate, amino acids, and various cytokines and growth factors on oocyte maturation and later embryonic development after *in vitro* fertilization (IVF) and parthenogenesis (PA) in pigs (Abeydeera *et al.* 2000; Sturmey and Leese, 2003; Hong and Lee, 2007; Mito *et al.* 2013).

Monosodium glutamate (MSG), a naturally present non-essential amino acid, is well known as a taste enhancer that is added frequently to foods, soups, and processed meats (Chang and Cha, 2001; Freeman, 2006; Ali *et al.*, 2014). Although MSG has been recognized as safe as a food ingredient for a long time, there are several studies reporting side effects of MSG such as MSG symptom complex including headache, sweating, chest pain, and nausea (Baad-Hansen *et al.*, 2010; Shimada *et al.*, 2013). MSG consists of 78% of glutamic acid and 22% of sodium (Samuels *et al*, 1999) and is an ingredient contained in meats, fishes, milk, and some vegetables (Winkel *et al.*, 2008). It has been

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reported that MSG exerts various physiological actions in cell metabolism in mammals. Glutamic acid, the main component of MSG, is used as a substrate for protein synthesis, involves in acid-base balance in kidney, and also acts as a precursor for neurotransmitter synthesis, nucleotide and nucleic acids, and glutathione (Newsholme *et al.*, 2003). Despite there are still controversy surrounding the safety or side effects of MSG as a flavor enhancer, dietary supplementation with MSG is generally recognized as safe in human, pig, and other animals (Boutry *et al.*, 2011; Brosnan and Brosnan, 2013; Rezaei *et al.*, 2014).

Effect of MSG on the reproductive function has been studied by several workers. Eweka and Om'Iniabohs (2011) reported that MSG at high doses might have some harmful effects on the oocytes of the ovaries and cause female infertility in adult Wistar rats. Oladipo *et al.* (2015) reported that MSG induced considerable changes in ovarian structure including degeneration of follicles, oocytes and medulla with vacuoles having blood congestion in Sprague-dawley rats. In another study, Spinacci *et al.* (2017) reported in pigs a beneficial effect of MSG on oocyte-sperm binding and fertilization *in vitro*. To date, a few studies are available on the effect of MSG on oocyte maturation and embryonic development in pigs. The objective of this study was to determine the effect of MSG during IVM and IVC on *in vitro* oocyte maturation and parthenogenetic development of PA embryos in pigs. To this end, immature pig oocytes were cultured under MSG treatment during *in vitro* maturation and nuclear maturation of oocytes, intraoocyte glutathione content, and developmental competence of parthenogenesis embryos were determined.

MATERIALS AND METHODS

1. Culture Media and Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. For IVM of immature pig oocytes, medium-199 (M-199) (Invitrogen, Grand Island, NY, USA) containing 10% (v/v) pig follicular fluid (Experiment 4) or porcine zygote medium (PZM)-4 containing 5.5 mM glucose and 0.1% (w/v) polyvinyl alcohol (PVA) were used (Experiments 1-3). These media were supplemented further with 0.91 mM pyruvate, 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 1 μg/ml insulin, 75 μg/ml kanamycin, and various concentrations of MSG according to the experimental design. The medium for *in vitro* culture (IVC) of PA and SCNT embryos was PZM-3 containing 0.3% (w/v) bovine serum albumin (BSA). The IVC medium was modified in this study by adding 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol (Lee *et al.*, 2014).

2. Oocyte Collection and IVM

Prepubertal gilt ovaries were obtained at a local abattoir. Follicular contents containing cumulus-oocyte complexes (COCs) were aspirated from superficial follicles of 3-8 mm in diameter. The COCs had multiple layers of cumulus cells were selected and washed in HEPES-buffered Tyrode's medium containing 0.05% (w/v) PVA (TLH-PVA). The COCs were placed into each well of a four-well culture dish (Nunc, Roskilde, Denmark) containing 500 μl of IVM medium enriched with 80 μg/ml follicle stimulating hormone (Antrin R-10, Kyoritsu Seiyaku, Tokyo, Japan) and 10 IU/ml human chorionic gonadotrophin (Intervet International BV, Boxmeer, Holland). The COCs were cultured at 39°C under humidified atmosphere of 5% CO2 and 95% air. After 22 h in the maturation culture, the COCs were washed properly and then cultured in hormone-free IVM medium for an additional 22 h and 20 h for PA and SCNT, respectively.

3. Experimental Design

In experiment 1, effects of various concentrations (0, 0.1, 0.5, and 5.0 mM) of MSG in a non-essential amino acids-free chemically defined PZM-4 on oocyte maturation and PA embryonic development were examined. Effect on MSG treatment during IVM as in Experiment 1 on intra-oocyte GSH contents and cumulus expansion of IVM oocytes were determined in Experiment 2. In Experiment 3, 0.1, 0.5, and 5.0 mM MSG were added to a chemically defined PZM-4 in the presence of 1% (v/v) minimum essential medium NEAA and effects of MSG on oocyte maturation and PA embryonic development were evaluated. Finally, effects of MSG treatment during IVC on PA embryonic development was assessed in Experiment 4.

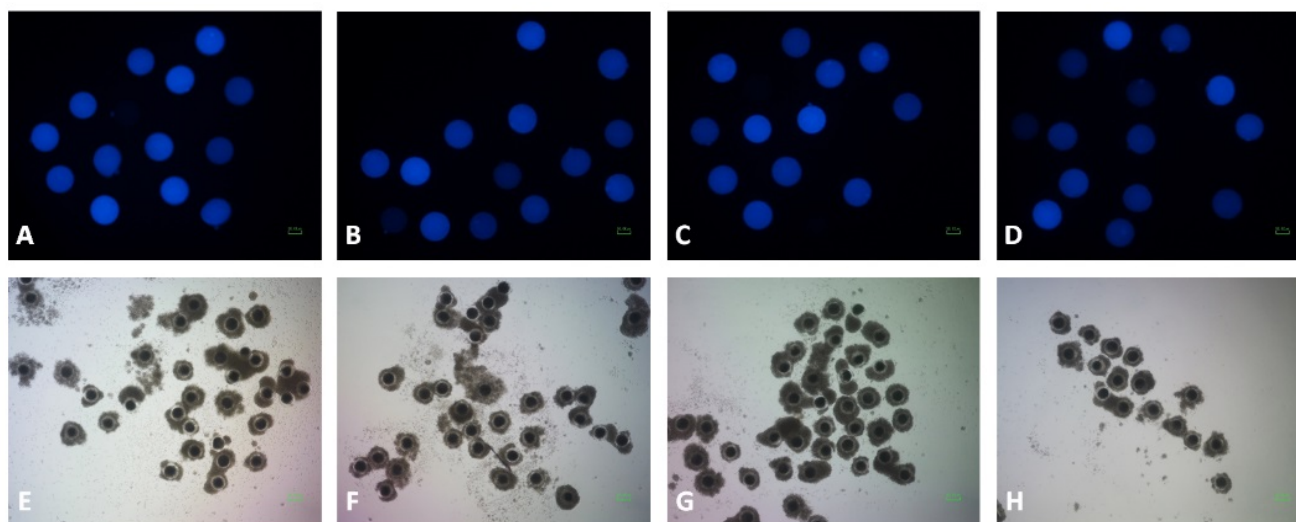

Fig. 1. Photomicrographic images of *in vitro*-matured porcine oocytes. Oocytes were matured in a non-essential amino acids-free porcine zygote medium-4 that was supplemented with 0.1% (w/v) polyvinyl alcohol, 5.5 mM glucose, and various concentrations of monosodium glutamate (MSG). Oocytes matured in maturation medium containing 0 (control, A and E), 0.1 (B and F), 0.5 (C and G), or 5.0 mM (D and H) MSG were examined for intra-oocyte glutathione (GSH) contents (A-D) and cumulus expansion (E-H). Oocytes were stained with CellTracker Blue to detect GSH contents.

4. Parthenogenetic Activation (PA) and *In Vitro* Culture of PA embryos

After IVM, oocytes were denuded from their cumulus cells by gentle pipetting in IVM medium containing 0.1% (w/v) hyaluronidase. Oocytes extruded the first polar body were selected and allocated to PA. Oocytes were placed in an electrode chamber and PA was induced by applying two pulses of 120 V/mm direct current for 60 µsec in a 280 mM mannitol solution that was supplemented with 0.05 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Then, activated oocytes were washed properly in IVC medium and incubated for 4 h in IVC medium containing 7.5 µg/ml. PA embryos were washed three times, placed in 30-µl IVC medium droplets under mineral oil, and cultured for 7 days at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

5. Determination of Intra-Oocyte GSH content and Cumulus Cell Expansion after IVM

GSH content in IVM oocytes was determined as previously described (Sakatani *et al.*, 2007) using Cell-Tracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen) to detect GSH as blue fluorescence (Fig. 1). A group of 10 to 12 oocytes per replicate were incubated for 30 min in TLH-PVA supplemented with 10 µM Cell-Tracker, washed properly and incubated for 30 min in a PZM-3 containing 0.3% (w/v) BSA at 39°C in the dark. Then, oocytes were washed, placed into 2-µL droplets of Dulbecco's phosphate-buffered saline (D-PBS) 0.1% (w/v) PVA, and observed for blue fluorescence under an epifluorescence microscope (TE-300; Nikon). The fluorescence intensities of oocytes were analyzed with the ImageJ software and normalized to control oocytes.

The extent of cumulus cell expansion after IVM was evaluated subjectively as previously reported (Vanderhyden *et al.*, 1990). Briefly, no expansion was scored as 0, minimum observable response with the cumulus cells in the outermost layer became round and glistening as 1, the expansion of outer cumulus cell layers as 2, the full expansion of all cumulus cell layers except corona radiata as 3, and the full expansion of all cumulus cell layers including corona radiata as 4 (Fig. 1).

6. Statistical Analysis

Statistical analyses were done using the Statistical Analysis System (version 9.3; SAS Institute, Cary, NC, USA). The data were analyzed by the general linear model procedure followed by the least square method when the treatments differed at $P < 0.05$. The percentage data such as embryo cleavage and blastocyst formation were arcsine transformed before analysis to maintain homogeneity of variance. The results are expressed as the mean ± standard error of the mean (SEM).

Table 1. Effect of monosodium glutamate (MSG) in a non-essential amino acids (NEAA)-free chemically defined medium on oocyte maturation and embryonic development after parthenogenesis (PA)

MSG (mM) in maturation medium*	% of oocytes that reached MII	No. of PA oocytes cultured†	% of embryos developed to		No. cells in blastocyst
			≥ 2-cell	Blastocyst	
0 (control)	86.3 ± 3.1	139	91.4 ± 2.6	25.6 ± 3.4[b]	38.0 ± 2.4
0.1	83.1 ± 3.0	138	92.8 ± 1.2	32.9 ± 4.6[ab]	40.8 ± 1.9
0.5	83.8 ± 2.1	144	93.1 ± 1.7	37.2 ± 5.8[ab]	40.1 ± 1.8
5.0	87.1 ± 1.8	148	93.4 ± 1.2	45.3 ± 6.2[a]	38.0 ± 2.0

† Four replicates.
*The maturation medium was NEAA-free porcine zygote medium (PZM)-4 containing 0.1% polyvinyl alcohol (PVA) and 5.5 mM glucose.
[ab]Values in the same column with different superscript letters are different (P < 0.05).

RESULTS

1. Effect of MSG in a Non-Essential Amino Acids (NEAA)-Free Chemically Defined Medium on Oocyte Maturation and PA Embryonic Development (Experiment 1)

When immature oocytes were matured in a chemically defined medium containing 0, 0.1, 0.5, and 5.0 mM MSG, nuclear maturation of oocytes (83.1-87.1%). After PA, blastocyst formation was significantly increased by 5.0 mM MSG treatment (45.3 ± 6.2%) during IVM compared to control (25.6 ± 3.4%) while embryo cleavage (91.4-93.4%) and mean number of cells in blastocyst (38.0-40.8 cells) were not altered by the treatment (Table 1).

2. Effect of MSG in a Non-Essential Amino Acids (NEAA)-Free Chemically Defined Medium on Intra-Oocyte GSH contents and Cumulus Expansion (Experiment 2)

The GSH contents (1.00 ± 0.06 and 0.89-0.98 pixels/oocyte for control and MSG-treated oocytes, respectively) cumulus expansion scores (1.50 and 1.20-1.28 for control and MSG-treated oocytes, respectively) of IVM oocytes that were untreated or treated with various concentrations of MSG are shown in Table 2. The GSH contents and cumulus expansion were not influenced by MSG treatment during IVM at all concentrations tested.

3. Effect of MSG in a Chemically Defined Medium Containing Non-Essential Amino Acids (NEAA) on oocyte maturation and PA embryonic development (Experiment 3)

As shown in Table 3, MSG treatment during IVM in the presence of NEAA did not alter nuclear maturation of oocytes (93.0-96.9%). After PA, embryo cleavage was significantly lower in 0.5 mM MSG group (89.3%) than in 0.1 mM MSG (94.6%). However, there was no difference in blastocyst formation (47.1 ± 2.3, 43.5 ± 4.0, and 50.4 ± 3.1% for 0.1, 0.5, and 5.0 mM MSG, respectively) and mean number of cells in blastocyst (32.2-33.7 cells) among the concentrations of MSG examined.

Table 2. Effect of monosodium glutamate (MSG) in a non-essential amino acids (NEAA)-free chemically defined medium on intra-oocyte glutathione (GSH) contents and cumulus cell expansion after IVM

MSG (mM) in maturation medium*	No. of MII oocytes examined for GSH	Relative level (pixels/oocyte) of GSH	No. of oocytes examined for cumulus expansion	Cumulus cell expansion score†
0 (control)	42	1.00 ± 0.06	77	1.50 ± 0.23
0.1	42	0.93 ± 0.06	77	1.25 ± 0.14
0.5	42	0.89 ± 0.06	76	1.28 ± 0.16
5.0	42	0.98 ± 0.06	82	1.20 ± 0.12

*Three replicates.
† Cumulus cell expansion was scored as 0 (no response), 1 (minimum observable response with the cells in the outermost layer of the cumulus become round and glistening), 2 (the expansion of outer cumulus cell layers), 3 (the expansion of all cumulus cell layers except corona radiata), and 4 (the expansion of all cumulus cell layers).

Table 3. Effect of monosodium glutamate (MSG) in a chemically defined medium containing non-essential amino acids (NEAA) on oocyte maturation and embryonic development parthenogenesis (PA)

MSG (mM) in maturation medium*	% of oocytes that reached MII	No. of PA embryos cultured†	% of embryos developed to		No. cells in blastocyst
			≥ 2-cell	Blastocyst	
0.1	96.2 ± 1.2	149	94.6 ± 1.1[a]	47.1 ± 2.3	32.6 ± 1.3
0.5	96.9 ± 1.5	151	89.3 ± 1.9[b]	43.5 ± 4.0	33.7 ± 1.3
5.0	93.0 ± 1.9	145	93.8 ± 0.7[ab]	50.4 ± 3.1	32.2 ± 1.3

† Four replicates.

* The maturation medium was porcine zygote medium (PZM)-4 containing 0.1% polyvinyl alcohol (PVA), 0.1% (v/v) NEAA, and 5.5 mM glucose.

ab Values in the same column with different superscript letters are different ($P < 0.05$).

4. Effect of MSG during IVC in a Non-Essential Amino Acids (NEAA)-Free Chemically Defined Medium on PA Embryonic Development (Experiment 4)

PA embryos were cultured in a NEAA-free chemically defined PZM-4 medium that was further supplemented with 0 (control), 0.1, 0.5, and 5.0 mM MSG to examine the effect of MSG on embryonic development. Treatment of embryos with 5.0 mM MSG (27.8 ± 4.9%) during IVC significantly decreased ($P < 0.05$) blastocyst formation of PA embryos compared to no treatment control (41.4 ± 1.9%). In addition, means cell number of blastocyst was also decreased in 5.0 mM MSG group (26.8 ± 1.1 cells/blastocyst) compared to 0.1 and 0.5 mM MSG groups (30.8 ± 1.2 and 31.1 ± 1.5 cells/blastocyst, respectively) (Table 4).

DISCUSSION

Reproductive biotechnologies such as IVF, intracytoplasmic sperm injection, and somatic cell nuclear transfer have been widely used to produce animals with specific purposes. In the utilization of these technologies, IVM oocytes are routinely used. Thus, the quality of IVM oocytes are essential factor for normal development of IVP mammalian embryos. In this study, we evaluated the effect of MSG, a routinely used taste enhancer in many kinds of foods, on oocyte maturation and subsequent *in vitro* development of PA embryos in pigs. We found that MSG showed differential effect on PA embryonic development depending on the time of treatment. MSG treatment at 5 mM concentration during IVM was beneficial for blastocyst formation of PA embryos whereas treatment of PA embryos with the same concentration of MSG for 7 days during IVC showed a deleterious effect on embryonic development.

In the pig follicular fluid, various amino acids including glutamate, glutamine, aspartate, glycine, arginine, alanine, leucine, lysine, proline, and valine (Jimena *et al.*, 1993; Hong and Lee, 2007). Of those, glutamate is the most plentiful amino acid irrespective of the follicular size and its concentration is approximately twice as much as those of other amino acids. In a previous study (Hong and Lee, 2007) examined the effect of various amino acids in a maturation

Table 4. Effect of monosodium glutamate (MSG) in *in vitro* culture (IVC) medium on embryonic development after parthenogenesis (PA)

MSG (mM) in IVC medium*	No. of PA embryos cultured†	% of embryos developed to		No. cells in blastocyst
		≥ 2-cell	Blastocyst	
0	170	90.6 ± 0.7	41.4 ± 1.9[a]	28.8 ± 1.2[ab]
0.1	171	87.8 ± 2.0	37.4 ± 3.4[ab]	30.8 ± 1.2[a]
0.5	171	88.4 ± 1.9	34.4 ± 2.6[ab]	31.1 ± 1.5[a]
5.0	170	90.0 ± 1.8	27.8 ± 4.9[b]	26.8 ± 1.1[b]

† Five replicates.

*The IVC medium was NEAA-free porcine zygote medium (PZM)-4 containing 0.1% (w/v) polyvinyl alcohol (PVA) and 5.5 mM glucose.

ab Values in the same column with different superscript letters are different ($P < 0.05$).

medium, amino acids such as arginine and alanine did not alter nuclear maturation of pig oocytes while blastocyst formation of IVF embryos. In this study, MSG treatment during IVM did not affect nuclear maturation of oocytes while 5 mM MSG improved blastocyst formation of PA embryos. This result was inconsistent with the previous finding 0.439 mM glutamic acid did not show beneficial effect on both oocyte maturation and IVF embryonic development. This discrepancy might be due to the different concentration of glutamate used. Hong and Lee (2007) determined the glutamate concentration as 0.439 mM in the pig follicular fluid and used that concentration of glutamate.

The extent of cumulus cell expansion and intra-oocyte GSH contents have been routinely used as an indicator to assess the quality of IVM oocytes (Eppig, 1982; Abeydeera *et al.*, 1998; De Matos and Furnus, 2000; Lee *et al.*, 2014) because *in vivo* oocytes shows full expansion of cumulus cell layer (Motlik *et al.*, 1986). It is well known that GSH protects oocytes from harmful effects of reactive oxygen species (Yoshida *et al.*, 1993; Maedomari *et al.*, 2007). In this study, although 5 mM MSG showed a beneficial effect on embryonic development, no effect of MSG treatment during IVM was observed in these parameters. There are several studies reported that the good cumulus cell expansion and high intra-oocyte GSH contents are important criteria of high quality of oocytes but does not guarantee better embryonic development (Marei *et al.*, 2012; Park *et al.*, 2016).

In contrast to the beneficial effect of MSG treatment during IVM, treatment of PA embryos with 5 mM MSG for 7 days during IVC inhibited blastocyst formation, especially at the 5 mM concentration. It was not clear how MSG acted and affected embryonic development in this study. Considering the concentration of glutamate in pig follicular was around 0.439 mM, the concentration of MSG used in this study (5.0 mM) was approximately 10 times higher than the follicular concentration. In addition, in contrast to IVM treatment for 44 h, PA embryos were treated for longer period (7 days) during IVC. Relatively longer period of treatment with high concentration of MSG might showed a deleterious effect. In summary, our results demonstrate that 5 mM MSG in a NEAA-free chemically defined maturation medium has positive effect on PA embryonic development while 5 mM MSG treatment during IVC is deleterious to PA embryonic development in pigs. Further studies would be needed to clarify the effect of MSG ingestion via foods on the change in glutamate concentration of body fluid such as blood, follicular fluid, and oviductal or uterine fluid and on the function of reproductive organs including follicular development, oocyte maturation, ovulation, and early embryonic development.

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