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Concentration and composition of free amino acids and osmolalities of porcine oviductal and uterine fluid and their effects on development of porcine IVF embryos

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Abstract

The concentration of free amino acids and the osmolalities in porcine oviductal (OF) and uterine fluids (UF) on Day 3 (D3) and Day 5 (D5) were measured by HPLC and Vapor Pressure Osmometer, respectively. Based on these measurements we designed new media based on PZM3 by modifying the amino acid composition and osmolality. The effectiveness of the modified PZM3 on the development of porcine IVF embryos was then investigated. A total of 24 free amino acids were measured, including 20 protein and 4 non-protein amino acids (β Alanine, Taurine, Ornithine and Citrulline). There was no significant difference in the total concentration of amino acids among D3OF (13.06 ± 3.63 mmol/L), D3UF (10.54 ± 5.16 mmol/L) or D5UF (10.23 ± 6.69 mmol/L). But the total concentration of amino acids in D5OF (5.89 ± 1.47 mmol/L) was significantly lower than the three fluids above. Some individual amino acids varied significantly depending on where they were collected from and the day. The blastocyst rates of porcine IVF embryos were not improved when embryos were cultured in PZM3 with amino acids at D3OF (PZM3-D3OF, $20.3 \pm 7.9\%$) or D5UF (PZM3-D5UF, $14.3 \pm 10.7\%$) concentrations or in PZM3-D3OF for the first 48 (20.5 ± 15.1), 72 (25.6 ± 10.4) and 96 (18.7 ± 10.0) hours and then transferred into PZM3-D5UF compared with PZM3 with Sigma amino acid solution (PZM3-SAA) ($30.8 \pm 9.1\%$). However, when IVF embryos were cultured in PZM3- D5UF, the average nuclear number per blastocyst (57.6 ± 8.3) was increased compared to PZM3-SAA (40.5 ± 3.5). The osmolalities in D3OF, D3UF, D5OF and D5UF were 318 ± 8 , 320 ± 32 , 321 and 293 ± 8 mOsM, respectively. When the IVF embryos were cultured in PZM3-SAA and PZM3-D3OF at a variety of osmolalities (150–360 mOsM), higher blastocyst rates were obtained at 270–300 mOsM in the PZM3-SAA group (24.6%–33.9%) and 270–290 mOsM in PZM3-D3OF group (22.4%–24.2%). The blastocyst rate gradually decreased when the osmolality was increased or decreased in both groups. When the embryos were cultured in PZM3-SAA at 330 mOsM for the first 72 hours and then transferred to 250 mOsM ($33.3 \pm 3.4\%$), the blastocyst rate was higher than original PZM3 ($21.2 \pm 2.2\%$) (288 mOsM).

Introduction

In vitro produced porcine embryos develop to the blastocyst stage at a lower rate and have fewer cells compared to in vivo produced embryos (Pollard *et al.* 1995; Macháty *et al.* 1998;

Niemann *et al.* 1983; Papaioannou & Ebert 1988). The main reason is that in vitro conditions have not been optimized. The goal of this experiment was to examine the physical and chemical composition of the female reproductive tract fluid and design new media according to these measurements.

Amino acids are thought to play an important role in mammalian embryo development. Exogenously supplied amino acids can improve the development of mammalian in vitro produced (IVP) embryos to the blastocyst stage, increase the total cell numbers (Takahashi & First 1992), and improve embryo quality and freezability through decreasing the lipid droplet size, protecting the cellular inner structure, and maintaining metabolism (Li *et al.* 2002; Gardner *et al.* 1994; Biggers *et al.* 2000). However, in most studies, amino acids in the medium for mammalian embryo culture are generally supplemented at concentrations used by Eagle (Eagle, 1959), which was designed based on the requirements for growth of mammalian fibroblasts rather than embryos. The free amino acid concentration and composition between bovine oviductal fluid and Eagle's formulation are obviously different. The total amount of Eagle amino acids (3.17mmol/L) is almost 10 times lower than that in bovine oviductal fluid (32.2mmol/L, Elhassan *et al.* 2001; 31.6mmol/L, Li *et al.* 2003), and the concentration of several amino acid, such as glycine, is about 140 times lower (Elhassan *et al.* 2001; Li *et al.* 2003). Some non-protein amino acids, such as taurine (Tau), exist in both bovine and murine reproduction tract fluid (Elhassan *et al.* 2001; Li *et al.* 2003; Harris *et al.* 2005) but are not contained in the Eagle formulation (Eagle 1959). Two research groups (McKiernan *et al.* 1995; Bavister & Arlotto 1990; Lane & Gardner 1997; Lane *et al.* 2001) have optimized the composition and concentration of amino acids in the culture media for hamster and mouse embryos. Ovine oviductal fluid concentrations of amino acids in vitro have been shown to enhance both ovine and bovine blastocyst development and hatching (Walker *et al.* 1996; Hill *et al.* 1997). Bovine oviductal and uterine fluid concentrations of amino acids also have been shown to improve bovine embryo development (Li *et al.* 2006). However, the amino acid concentration in the reproductive tract can vary between species. While there are a few reports regarding the composition and concentration of free amino acids in female reproductive tract in pig (Iritani *et al.* 1974, Guérin *et al.* 1995), they were not done on different days of pregnancy.

The osmolality of the medium is an important factor for mammalian embryo development. The osmolality of mouse oviductal fluid has been calculated to be above 340 mOsM (Borland *et al.* 1977; Van Winkle *et al.* 1990). A similarly high osmolality is predicated for human fallopian tube fluid by electron probe measurements (Borland *et al.* 1988). The osmolality of oviductal fluid in mouse had been directly determined to be 290–300 mOsM (Collins & Baltz 1999). Interestingly, while the physiological osmolality is very high, the most successful media for culture mammalian embryos from the zygote to blastocyst stage have very low osmolalities, in the range of 250–280 mOsM (Liu & Foote 1995; Liu & Foote 1996; Brinster 1965). Osmolality greater than about 300 mOsM is detrimental to the mammalian embryo (Van Winkle *et al.* 1990; Hay-Schmidt 1993; Miyoshi *et al.* 1994; Hadi *et al.* 2005). It appears that the reason the embryo can survive the high physiological osmolality in vivo is that there are amino acids serving as “organic osmolytes”, such as glycine (Dawson & Baltz 1997) glutamine (Biggers *et al.* 1993) and β -alanine (Hammer & Baltz 2003), in the female reproductive tract. If any of a number of “organic osmolytes” is included in the culture medium, the embryo will develop at significantly higher osmolalities than in the absence of such compounds in vitro (Van Winkle *et al.* 1990; Dawson & Baltz 1997). These osmoprotectants can be imported into the cell to provide intracellular osmotic support and maintain cell volume against exposure to high osmolality (Hammer & Baltz 2003). However, besides some special amino acid such as glycine (Hadi *et al.* 2005), the effects of most amino acids when they are included in the medium at the physiological levels have not been investigated.

In this study, we first measured the concentration of free amino acids and the osmolalities in porcine oviductal and uterine fluids on day 3 and day 5 after estrus. Based on these measurements and used PZM3 without amino acids as basic medium (Yoshioka *et al.* 2002), we designed new media. The effectiveness of the modified media on the development of porcine IVF embryos was then investigated.

Materials and Methods

All chemicals were purchased from Sigma, St. Louis, MO USA unless otherwise stated.

Experimental design

This study consisted of five parts: 1. Determine the concentrations and composition of free amino acids in porcine D3OF, D3UF, D5OF and D5UF. 2. Make new media based on supplementation with amino acids at D3OF and D5UF concentrations by using PZM3 (amino acid free) (Yoshioka *et al.* 2002) as the basic medium (Abbreviated as PZM3-D3OF and PZM3-D5UF). We investigated the effects of these media on porcine IVF embryo development, PZM3 with SAA (2% v/v Basal Medium Eagle amino acids (Sigma B6766) and 1% v/v Minimum Essential Medium nonessential amino acids (Sigma M7145) served as control (Abbreviated as PZM3-SAA). 3. We measured the osmolality of porcine D3OF, D3UF, D5OF and D5UF. 4. We determined the effects of amino acids at D3OF concentration on protection of IVF embryo from higher or lower osmolality damage by culturing IVF embryos in PZM3-SAA and PZM3-D3OF at a variety of osmolalities (150, 250, 270, 290, 300, 330 or 360 mOsM). 5. According to the results of experiment 3 (the osmolality of D5UF tended to be higher than that of D3OF) and 4 (PZM3-D3OF and PZM3-SAA had same protective effects on embryo development against lower or higher osmolalities), we chose PZM3-SAA to determine the effects of transfer of IVF produced embryos from different osmolalities 270, 290, 300, 330 and 360 mOsM on day 3 after IVF to 250 or 270 mOsM on embryo development.

Collection of oviductal and uterine fluid

Four mated gilts on day 3 (Gilt 492, 523, 525 and 532) and day 5 (Gilt 505, 508, 513 and 535) after estrus (Day 0 is the time of behavioral estrous) were killed and their whole reproductive tracts were transferred in 5 min to the laboratory at 0–4°C. The oviduct and uterus were separated and then gently squeezed from one end to the other by using forceps. The fluid was collected in an eppendorf tube or 15 ml conical tube and centrifuged at $9,300 \times g$ for 5 min. Twenty μ l of each sample was desiccated using a speed vacuum and then sent to Texas A&M University for free amino acid test. The leftover volume was stored at –20°C until osmolality analysis. Ten μ l of the sample was used for osmolality analysis. The oviductal fluid of Gilts 532, 505, 508 and 535 was enough only for the amino acid test, so they were not tested for osmolality.

Amino acid and osmolality analysis

The samples were analyzed on a Hewlett Packard AminoQuant System at Texas A&M University to measure the free amino acid concentration. This technique includes automated precolumn derivitization of the hydrolyzed primary amino acids with OPA and the secondary amino acids with 9-fluoromethyl-chloroformate (FMOC). Derivatized amino acids were separated by reverse phase HPLC on the HP 1090L and detected by Fluorescence (FLD). Derivatized amino acids were eluted from a narrow bore, (2.1*200mm), (Hypersil AA-ODS), 5 μ m reverse phase column (Agilent, part # 79916AA-572). Solvent A consisted of a 20 mmol/L sodium acetate buffer with 0.018% v/v triethylamine (Fluka 90338), 0.05 mmol/L EDTA and 0.3% tetrahydrofuran (Fluka 87363) adjusted to pH 7.2 with weak acetic acid. Solvent B was a 20% 100 mmol/L sodium acetate buffer with 40% acetonitrile and 40% methanol. The working gradient begins at 0 minutes at 100% A at 0.45 ml/min and goes to 60% B over 17

minutes. Primary amino acids (tagged with OPA) were detected at excitation/emission 340/450 nm by fluorometric detector and secondary amino acids (tagged with FMOC) at 266/305 nm.

Osmolality of the sample was determined by using a Vapor Pressure Osmometer (Model 5520, Wescor INC, Utah) according to manufacture's instructions.

Collection of porcine oocytes, in vitro maturation and fertilization

Ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl at 30–35°C. Cumulus oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) with a syringe and an 18-gauge needle. Approximately 50 oocytes were cultured in 500 µl maturation medium TCM 199 (Gibco, 31100035, Grand Island, NY) with 0.1% PVA, 3.05 mmol/L glucose, 0.91 mmol/L sodium pyruvate, 0.57 mmol/L cysteine, 0.5 µg/mL LH, 0.5 µg/mL FSH, 10 ng/mL epidermal growth factor, 75 µg/mL penicillin and 50 µg/mL streptomycin, for 40–44 h at 38.5°C, 5% CO₂ in humidified air. After maturation the cumulus cells were removed from the oocytes by vortexing for 4 min in TL-HEPES supplemented with 0.1% PVA and 0.1% hyaluronidase. For IVF, 30–40 denuded oocytes with a polar body were transferred to 50 µL of IVF medium. One 0.1 mL frozen semen pellet was thawed into 3 mL sperm wash (PBS; Gibco) with 1 mg/mL BSA at 39°C and then layered on to 60% percoll in PBS for centrifugation (600×g, 10min). After centrifugation, the pellet was transferred into 10 mL sperm wash and centrifuged (600×g, 5 min) for a second wash. The supernatant was removed and the sperm pellet was suspended at 1×10^6 sperm/mL in IVF medium. The sperm suspension (50 µL) was added to a 50 µL IVF drop with oocytes and cultured at 38.5°C, 5% CO₂ in air for 4 h. The sperm wash was PBS (calcium and magnesium free) with 1 mg/ml BSA, 75 µg/mL penicillin and 50 µg/mL streptomycin. The IVF medium was a modified Tris-buffered medium with 113.1 mmol/L NaCl, 3 mmol/L KCl, 7.5 mmol/L CaCl₂, 5 mmol/L sodium pyruvate, 11 mmol/L glucose, 20 mmol/L Tris, 2 mmol/L caffeine, and 2 mg/mL of BSA.

In vitro culture of IVF embryos

After IVF, the presumptive zygotes were cultured in a variety culture media. The amino acid concentration in day 3 oviductal fluid and day 5 uterine fluid is shown in Table 1. Basal PZM3 culture medium was supplemented with amino acids corresponding to the values presented in Table 1 for PZM3-D3OF and PZM3-D5UF, respectively. NaCl was used to adjust the osmolality of medium. The concentration of NaCl in media with different osmolalities is shown in Table 5 and Table 6. To make PZM3-D3OF and PZM3-D5UF, four 20× amino acid stock solutions, 2× PZM3 stock solution (excluded NaCl) and 10× NaCl stock solution (1.08mol/L) were made. The 1st amino acid stock solution included Asp, Glu, Tuar, Tyr and cystine and 0.1 N NaOH was used as solvent. The 2nd included His, Cit, Arg and Lys and 0.1N HCl was used as solvent. The 3rd included Asn, Ser, Gln, Gly, Thr, Cysteine, β Ala. The 4th included Ala, Val, Met, Trp, Phe, Ile, Orn, Leu and Pro. Both 3rd and 4th used distilled water as solvent. The final culture solutions were made by diluting the stock solutions with water. The different osmolarities were obtained by added different volume NaCl stock solution. Twenty to 30 embryos were cultured in 500 µL of medium. Cleavage was observed 48 h after IVF and the blastocyst rates and nuclei number of the blastocysts was observed on Day 7 after IVF. Hoechst 33342 (4 µg/mL) was used to stain the nuclei of the blastocysts and the stained blastocysts were examined by epifluorescent microscopy.

Statistic analysis

The t-test (SAS software) was used to compare the cleavage and blastocyst rates, the number of nuclei in the different groups of embryos, the each and total amino acid concentrations and the osmolalities in the different fluids.

Results

The concentration and composition of free amino acids in porcine D3OF, D3UF, D5OF and D5UF

A total of 24 free amino acids were identified, including 20 protein and 4 non-protein amino acids (ornithine, taurine, β -alanine and citrulline). There was no significant difference in the total concentration of amino acids among D3OF (13.06 ± 3.63 mmol/L), D3UF (10.54 ± 5.16 mmol/L) and D5UF (10.23 ± 6.69 mmol/L). But the concentration of amino acids in D5OF (5.89 ± 1.47 mmol/L) was significantly lower than the three fluids above ($P < 0.05$). All 4 fluids were significantly different in the total concentration of amino acids compared to the formulation of PZM3 with SAA ($P < 0.05$). Some individual amino acid, such as Ser, His Thr and so on, varied significantly depending on where they were collected from and the day. The concentration of each amino acid was shown in Table 1.

The effect of amino acids at oviductal or uterine fluid concentration on the development of porcine IVF embryos

The blastocyst rates of porcine IVF embryos were not improved when embryos were cultured in PZM3-D3OF (20.3 ± 7.9) or PZM3-D5UF (14.3 ± 10.7) or in PZM3-D3OF for the first 48 (20.5 ± 15.1), 72 (25.6 ± 10.4) and 96 (18.7 ± 10.0) hours and then transferred into PZM3-D5UF compared with PZM3-SAA (30.8 ± 9.1). However, when IVF-derived embryos were cultured in PZM3-D5UF, the average nuclear number per blastocyst (57.6 ± 8.3) was significantly higher compared to that cultured in PZM3-SAA (40.5 ± 3.5) (Table 2).

Osmolalities of D3OF, D3UF, D5OF and D5UF

The osmolality of D3OF, D3UF, D5OF and D5UF was 318 ± 8 , 320 ± 32 , 321 and 293 ± 8 mOsM, respectively. The osmolality of D3OF tend to be higher than that of D5UF (Table 3).

The effects of physiological levels of amino acids at D3OF concentration on protection of IVF embryo from higher or lower osmolality damage

When the IVF embryos were cultured in PZM3-SAA and PZM3-D3OF at a variety of osmolalities (150, 250, 270, 290, 300, 330 or 360 mOsM), higher blastocyst rates were obtained at 270–300 mOsM in the SAA group (24.6%–33.9%) and 270–290 mOsM in D3OF group (22.4%–24.2%). The blastocyst rate gradually decreased when the osmolality increased or decreased in both D3OF and SAA groups (Table 4).

The effects of transfer of IVF embryos from high to low osmolality on embryo development

The IVF embryos were cultured in PZM3-SAA at 270, 290, 300, 330 or 360 mOsM, respectively, for the first 3 days and then transferred to 250 or 270 mOsM. The original PZM3 (288 mOsM) served as control. When the embryos were cultured in high osmolality (330 mOsM) then transferred to low osmolality (250 mOsM) ($33.3 \pm 3.4\%$), the percentage blastocyst was higher than control ($21.2 \pm 2.2\%$) ($p < 0.05$). However, when the embryos were cultured in 360 mOsM then transferred to 270 mOsM, the embryo quality (number of nuclei) (30.0 ± 3.7) decreased significantly compared to control (46.6 ± 8.4) ($p < 0.05$) (Table 5).

Discussion

The concentration and composition of free amino acids in the porcine female reproductive tract were extremely different compared to the formulation of PZM3 with SAA. The total concentration of free amino acids in D3OF (13.1 mmol/L) and D5UF (10.2 mmol/L) were 4 and 3 times higher than that in SAA (3.17 mmol/L). Several amino acids in D3OF such as Gly (4.1 mmol/L), Arg (1.70 mmol/L), Ala (1.18 mmol/L) and Glu (1.03 mmol/L) are 41, 17, 12,

and 10 times higher, respectively, than that in SAA. Gly (3.8 mmol/L) and Glu (1.0 mmol/L) in D5UF are 38 and 10 times higher. Four non-protein amino acids (β Ala, Tau, Orn and Cit) existed in porcine OF and UF, but are not included in the Sigma formulation. The concentration of Tau is as high as 3.2 mmol/L in D5UF. Since amino acids are important for normal embryo metabolism, their inclusion in culture medium at appropriate concentrations should be beneficial to embryo development.

Although some individual amino acids, such as Ser, His Thr and so on, varied significantly depending on where they were collected from and the day, there is no significant difference in total amino acid concentration between D3OF and D5UF. The utilization of amino acids by the embryo is flexible (Gardner 1998), when individual amino acids of exogenous are limited, other amino acids may be converted to the required kind of amino acid and then utilized. For example, Gly may be converted to Ser and Ala after being taken up by a mouse embryo (Hobbs & Kaye 1985). When the embryos were cultured by two-steps, in PZM3-D3OF for the first 48, 72, and 96 hours and then transferred into PZM3-D5UF, both blastocyst development rate and embryo quality were not affected. All of these results indicate that the amino acid environment for porcine early embryo development tends to be stable. Nevertheless, physiological concentrations of amino acids have not improved blastocyst development rate compared with SAA. The reasons might be that the amino acids composition and concentration in SAA can support the embryo to develop in vitro well or that the beneficial effect of physiological levels of amino acids on embryo development might need the cooperation of other ingredients which also need to be mimicked to a physiological level.

Exogenous supplements of free amino acids can affect the osmolality of the medium. In this study, the osmolalities of medium were 290–295 mOsm and 275–280 mOsm, respectively, when amino acids were added to same basic PZM3 at D3OF and SAA concentrations. Some studies indicate that both osmolality and NaCl concentration can affect embryo development, especially at the zygote stage (Li & Foote 1996; Collins & Baltz 1999). However, mouse embryo development is inhibited by hyperosmotic media, whether produced by adding NaCl or raffinose. This demonstrated that it is not high NaCl concentration, but that the osmolality itself is detrimental to the embryo (Dawson & Baltz 1997). In this study, in order to investigate the effect of different osmolalities and physiological levels of amino acids on embryo development, the IVF embryos were cultured in PZM3 with amino acids at SAA or D3OF concentration at a variety of osmolalities (150, 250, 270, 290, 300, 330 or 360 mOsm) by using NaCl to adjust osmolality of medium. Higher blastocyst rates were obtained at 270–300 mOsm in the SAA group and 270–290 mOsm in D3OF group, which are similar to the original PZM3 (288 mOsm). Because some amino acids can be used as organic osmolytes, the D3OF, which included a more appropriate composition and concentration of amino acids, was expected to have a stronger ability to protect embryos from higher or lower osmolality damage, but it did not. The blastocyst rates gradually decreased when the osmolality increased or decreased in both D3OF and SAA groups. These physiological levels of amino acids have not been shown to protect embryos from higher or lower osmolality damage. These results demonstrate that the protective effect of amino acids, even at physiological levels, was limited, and the amino acids that existed in the Sigma formulation, such as glycine and proline (0.1 mmol/L), are not too far below the affinity constant for the relevant transport system and enough to protect the embryo against higher or lower osmolality. So the relative higher concentration of amino acids in D3OF and the relative lower concentrations of amino acids in SAA had almost same ability to protect the embryo against detrimental osmolalities. In addition, the osmolytes glutamine and hypotaurine have been included in PZM3 formulation at 1 mmol/L and 5 mmol/L concentrations which have been shown to be effective in protecting against hyper osmolality (Dawson & Baltz 1997).

Based on the percentage blastocyst, both parthenogenetic (Nguyen *et al.* 2003) and IVF embryos can be cultured in media with higher osmolalities (parthenogenetic embryo: 280–320mOsM; IVF embryo: 330mOsM) for the first 2–3 days after electric activation and IVF, and then in the media with lower osmolalities (parthenogenetic embryo: 220–270mOsM; IVF embryo: 250 mOsM). These results are consistent with the higher osmolality of D3OF and the lower osmolality of D5UF. Increased osmolality stimulated glycine accumulation in mouse oocyte and embryo, however, the ability of increased osmolality (350 mOsM) to stimulate glycine accumulation was lost by the 4-cell stage, even of the embryos continued to be cultured in high osmolality condition (350 mOsM), and the GLYT1-mediated accumulation had not disappeared until at the morula stage (Rudraraju & Baltz 2006). After the embryo stops absorbing the organic osmolytes glycine, the continued supply of higher osmolality might be detrimental to the embryo's development. Therefore, culturing porcine embryos in a higher osmolality (330mOsM) first, and then transferring into lower osmolality (250mOsM) in time appears to be beneficial for porcine embryo culture. However, increased osmolality (360mOsM) for the first 72 hrs culture and then 270 mOsM, which was not low enough, negatively affected the embryo quality by reducing the average nuclei number to 30.0 ± 3.7 .

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Table 1

The concentration of free amino acids in porcine oviductal fluid, uterine fluid and PZM3 (mmol/L).

Amino acid	Day3 Oviductal Fluid		Day5 Oviductal Fluid		Day3 Uterine Fluid		Day5 Uterine Fluid		PZM3 with Sigma amino acid solution (BME and MEM)
	Mean ± SEM		Mean ± SEM		Mean ± SEM		Mean ± SEM		
Aspartic acid, ASP	0.2365 ± 0.0233 a		0.1063 ± 0.0241 a		0.1739 ± 0.0667 a		0.1286 ± 0.0533 a		0.10
Glutamic acid, GLU	1.0318 ± 0.1991 a		0.4826 ± 0.1324 a		0.3954 ± 0.0548 a		0.9983 ± 0.7053 a		0.10
Asparagine, ASN	0.0178 ± 0.0147 a		0.0250 ± 0.0107 a		0.0267 ± 0.0199 a		0.0128 ± 0.0071 a		0.10
Serine, SER	0.5401 ± 0.0509 ab		0.2359 ± 0.0477 c		0.7704 ± 0.1191 a		0.3267 ± 0.0954 bc		0.10
Glutamine, GLN	0.2361 ± 0.0446 a		0.1215 ± 0.0512 a		0.1637 ± 0.0259 a		0.1508 ± 0.0726 a		1.00
Histidine, HIS	0.1352 ± 0.0073 a		0.0525 ± 0.0078 b		0.0855 ± 0.0336 ab		0.0416 ± 0.0078 b		0.05
Glycine, GLY	4.0713 ± 1.3784 a		1.7012 ± 0.2295 a		5.3570 ± 2.6163 a		3.8132 ± 2.5416 a		0.10
Threonine, THR	0.2884 ± 0.0319 a		0.1416 ± 0.0283 b		0.1320 ± 0.0752 b		0.0658 ± 0.0166 b		0.20
Cysteine	0.2197 ± 0.0389 a		0.0998 ± 0.0277 b		0.1171 ± 0.0401 b		0.0540 ± 0.0275 b		0
Citrulline, CIT	0.1192 ± 0.0304 a		0.0580 ± 0.0229 a		0.0846 ± 0.0385 a		0.0535 ± 0.0377 a		0
β-Alanine, β-ALA	0.1007 ± 0.0343 a		0.0449 ± 0.0185 a		0.0710 ± 0.0328 a		0.0731 ± 0.0310 a		0
Arginine, ARG	1.6999 ± 0.5158 a		0.4869 ± 0.2966 b		0.5933 ± 0.3455 b		0.2223 ± 0.1477 b		0.10
Alanine, ALA	1.1799 ± 0.4215 a		0.7650 ± 0.2267 a		1.1631 ± 0.9846 a		0.4852 ± 0.3208 a		0.10
Taurine, TUAR	0.5894 ± 0.4277 a		0.5077 ± 0.1248 a		0.3055 ± 0.2414 a		3.1703 ± 2.4175 a		0
Tyrosine, TYR	0.1541 ± 0.0128 a		0.0600 ± 0.0085 b		0.0637 ± 0.0377 b		0.0362 ± 0.0126 b		0.10
CYSTINE	0		0		0		0		0.05
Valine, VAL	1.0491 ± 0.2614 a		0.4674 ± 0.1220 b		0.3129 ± 0.1109 b		0.1755 ± 0.0407 b		0.20
Methionine, MET	0.0901 ± 0.0107 a		0.0339 ± 0.0057 b		0.0423 ± 0.0211 b		0.0166 ± 0.0073 b		0.05
Tryptophan, TRP	0.0294 ± 0.0014 a		0.0122 ± 0.0015 a		0.0285 ± 0.0024 a		0.0296 ± 0.0159 a		0.02
Phenylalanine, PHE	0.1638 ± 0.0215 a		0.0642 ± 0.0092 b		0.1320 ± 0.0380 ab		0.0851 ± 0.0284 ab		0.10
Isoleucine, ILE	0.1715 ± 0.0148 a		0.0667 ± 0.0097 b		0.0779 ± 0.0475 b		0.0404 ± 0.0187 b		0.20
Ornithine, ORN	0.0066 ± 0.0023 a		0.0011 ± 0.0009 a		0.0070 ± 0.0029 a		0.0041 ± 0.0020 a		0
Leucine, LEU	0.3593 ± 0.0271 a		0.1418 ± 0.0209 b		0.1874 ± 0.0901 b		0.1029 ± 0.0438 b		0.20
Lysine, LYS	0.3606 ± 0.0544 a		0.1163 ± 0.0232 b		0.1364 ± 0.0745 b		0.0474 ± 0.0108 b		0.20
Proline, PRO	0.2060 ± 0.0058 a		0.0998 ± 0.0193 b		0.1152 ± 0.0403 b		0.0955 ± 0.0229 b		0.10
Total	13.0567 ± 3.6310 a		5.8923 ± 1.4697 b		10.5427 ± 5.1598 a		10.2295 ± 6.6851 a		3.17 c

Values with different letters within the same row differ (p<0.05).

Table 2
The effect of amino acids at oviductal or uterine fluid concentrations on the development of porcine IVF embryos.

Treatment groups	No. of oocyte	Cleaved (Mean % ± SEM)	Blastocyst (Mean % ± SEM)	Nuclei No. of blastocyst(Mean ± SEM)
D3OF	79	61(77.2±0.7)	16(20.3±7.9)	37.8±6.9 ^{ab}
D5UF	77	60(77.9±7.3)	11(14.3±10.7)	57.6±8.3 ^a
D3OF 2day~ D5UF	78	65(83.3±5.9)	16(20.5±15.1)	42.6±5.8 ^{ab}
D3OF 3day~ D5UF	78	63(80.8±4.8)	20(25.6±10.4)	42.0±3.4 ^b
D3OF 4day~ D5UF	75	64(85.3±1.3)	14(18.7±10.0)	34.3±3.0 ^{ab}
Control	78	60(76.9±7.5)	24(30.8±9.1)	40.5±3.5 ^b

Values with different superscripts within the same column differ (p<0.05).

Data represents three replicates.

Table 3
The osmolalities of porcine oviductal and uterine fluid

Sample	Osmolality (mOsm)			Mean $\pm$ SEM
	Gilt No. 492	Gilt No. 523	Gilt No. 525	
Day 3 oviductal fluid	331	317	305	318 $\pm$ 8
Day 3 uterine fluid	264	406	328	320 $\pm$ 32
Day 5 oviductal fluid	Gilt No. 505	Gilt No. 508	Gilt No. 513	321*
	N/A	N/A	321	
Day 5 uterine fluid	312	281	298	293 $\pm$ 8

* Only one replicate.

Table 4
Effects of variety of osmolalities in PZM3 with amino acid at SAA or D3OF concentration on the development of porcine IVF embryo

Concentration of NaCl (mM)	Anticipated/Factual osmolality		PZM3 containing sigma amino acid stock solution		Concentration of NaCl (mM)	Anticipated/Factual osmolality		PZM3 containing D3 OF concentrations of amino acids	
			No. of oocyte	Cleaved (Mean % $\pm$ SEM)	Blastocyst (Mean % $\pm$ SEM)			No. of oocyte	Blastocyst (Mean % $\pm$ SEM)
46.5	150/150		93	35 (37.6 $\pm$ 10.6) a	0 (0) a			92	29 (31.5 $\pm$ 1.0) a
71.7	200/203		89	71 (79.8 $\pm$ 7.7) b	8 (9.0 $\pm$ 5.9) ab			94	68 (72.3 $\pm$ 15.8) b
95.1	250/249		116	87 (75.0 $\pm$ 4.8) bcd	23 (19.8 $\pm$ 8.0) cd			117	80 (68.3 $\pm$ 7.6) b
108.0	270/274		115	101 (87.8 $\pm$ 1.7) e	39 (33.9 $\pm$ 7.0) e			99	74 (74.7 $\pm$ 8.0) b
115.0	288/287		120	100 (83.3 $\pm$ 4.1) de	33 (27.5 $\pm$ 4.9) de			107	76 (71.0 $\pm$ 8.2) b
121.1	300/299		122	94 (77.0 $\pm$ 5.2) bcd	30 (24.6 $\pm$ 4.7) de			101	67 (66.3 $\pm$ 6.2) b
136.2	330/288		119	97 (81.5 $\pm$ 4.2) cde	23 (19.3 $\pm$ 4.9) cd			101	62 (64.2 $\pm$ 12.2) b
151.3	360/357		131	97 (74.0 $\pm$ 5.7) bc	12 (9.2 $\pm$ 4.8) bc			109	70 (64.2 $\pm$ 5.6) b

Values with different superscripts within the same column differ (p<0.05).

Data represents six replicates.

Table 5
Effects of variety of osmolalities in PZM3 with SAA on the development of porcine IVF embryo

Osmolality of the first 72 h culture	Osmolality after 72 h culture	No. of oocytes	Cleaved (Mean %± SEM)	Blastocyst (Mean %± SEM)	Nuclei No. of (Mean ± SEM)
270±2	250±2	98	80 (81.6±2.8)	29 (29.6±5.3) ^{ab}	38.2±2.5 ^{abc}
	270±2	100	83 (83.0±3.8)	21 (21.0±4.1) ^a	41.9±7.3 ^{abc}
290±2	250±2	101	85 (84.2±7.1)	26 (25.7±5.2) ^{ab}	48.2±11.2 ^a
	270±2	97	84 (86.6±6.6)	28 (28.9±6.6) ^{ab}	37.3±1.7 ^{abc}
300±2	250±2	100	87 (87.0±1.8)	29 (29.0±3.4) ^{ab}	34.3±3.7 ^{bc}
	270±2	98	85 (86.7±2.1)	27 (27.6±3.1) ^{ab}	40.0±3.3 ^{abc}
330±2	250±2	108	92 (85.2±1.3)	36 (33.3±3.4) ^b	33.5±2.3 ^{bc}
	270±2	110	95 (86.4±2.6)	35 (31.8±1.7) ^{ab}	36.3±4.7 ^{bc}
360±2	250±2	106	87 (82.1±1.9)	33 (31.1±4.5) ^{ab}	32.9±2.7 ^{bc}
	270±2	107	86 (80.4±2.2)	28 (26.2±3.0) ^{ab}	30.0±3.7 ^c
288±2	288±2	104	81 (77.9±4.7)	22 (21.2±2.2) ^a	46.6±8.4 ^{ab}

Values with different superscripts within the same column differ (p<0.05). Data represents four replicates.