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Effect of MEM Vitamins Supplementation of *In vitro* Maturation Medium and *In vitro* Culture Medium on the Development of Porcine Embryos

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ABSTRACT : This study was carried out to examine the influence of minimum essential medium (MEM) vitamins supplementation to *in vitro* maturation medium and *in vitro* culture medium on the development of porcine embryos. Porcine embryo development was investigated following cultivation in both *in vitro* maturation and culture medium with the supplementation of MEM vitamins (0, 0.1, 0.2 and 0.4%) using immature oocytes collected from the ovary of prepubertal gilts. Embryo development was observed and the total cell number in each blastocyst generated under the culture conditions was quantified following supplementation of the medium. The embryonic development rate of the blastocyst and hatched blastocyst was higher, but not significantly so, when 0.4% MEM vitamins were supplemented to the *in vitro* maturation medium of the porcine oocyte. Interestingly, the total number of cells in the blastocyst was significantly higher in the *in vitro* maturation MEM vitamins supplemented group compared to either the untreated group or the group which had MEM vitamins supplemented to both *in vitro* maturation and *in vitro* culture medium simultaneously ($p<0.05$). Therefore, the supplementation of 0.4% MEM vitamins to the *in vitro* mature medium has a beneficial effect on the embryonic development of *in vitro* produced blastocysts derived from the immature porcine oocytes. (**Key Words :** Porcine, Embryo Development, Blastocyst, Vitamins)

INTRODUCTION

In vitro production (IVP) of porcine embryos is used not only to study the generation process of mammalian embryogenesis but also widely used for both veterinary animal husbandry and biotechnology such as in the production of individuals containing superior genetic characters by embryo transplantation, the development of micro manipulation techniques, and stem cell studies.

Over the past several years, researchers have achieved *in vitro* fertilization of porcine oocytes (Iritani et al., 1978) and have succeeded in birthing piglets by *in vitro* embryo transplantation (Mattioli et al., 1989). Three main factors which determine the success of *in vitro* produced mammalian embryos are the *in vitro* maturity level of immature oocytes, the *in vitro* fertilization process and the method of embryos *in vitro* culture. Although multiple babies can be produced using *in vitro* produced blastocysts in other mammalian animals (Kikuchi et al., 2002), embryo transplantation technology is not as well established in the case of porcine. For example, cytoplasm maturation along with nucleus maturation is not well described in porcine, compared with other animals. Therefore, there are still many difficulties in securing high quality embryos due to the developmental retardation and stoppage (Camous et al., 1984; Heyman et al., 1987) in the 4-cell stage during *in vitro* culture because of unstable *in vitro* maturation (Wang et al., 1997), unstable male pronucleus generation (Motlik et al., 1984) and polyspermy during *in vitro* fertilization.

Several new media have been developed such as mWM, beltsville embryo culture medium-3 (BECM-3) consisting of simple minerals, an energy source, amino acids, a pH buffer, trace elements and antibiotics (Dobrinsky et al., 1996), low concentrated sodium lactate and NCSU23 with glucose (Petters and wells, 1993; Abeydeera et al., 2000; Abeydeera et al., 2001) and free-glucose and porcine zygote medium-3 (PZM-3) containing various amino acids (Yoshioka et al., 2002). In addition, improvements have been made to the *in vitro* production methods of generating porcine oocytes by co-culture with somatic cells (Xu et al., 1992), a static/perifusion culture system (Lim et al., 1997),

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the supplementation of growth factors (May et al., 1988; Chang et al., 2000), and the supplementation of antioxidants (Ali et al., 2003) since active oxygen in the medium is harmful for embryo development (Legge et al., 1991). In spite of these developments, less than 25% of total oocytes produce blastocysts following the *in vitro* culture of porcine oocytes (Stoke et al., 2005) with very low reproducibility and this leads to a remarkably low pregnancy rate following embryo transplantation (Prather and Day, 1998).

Among the alterations of *in vitro* culture conditions, it was reported that the supplementation of water-soluble minimum essential medium (MEM) vitamins to the *in vitro* culture medium is effective for the blastocole expansion of the blastocysts of rabbits (Kane, 1988). Furthermore, in the case of the hamster, it was also reported that MEM vitamins supplementation increased the embryo development rate of blastocysts and the hatched blastocysts from the 8-cell stage (Mishra and Seshagiri, 1998). However, MEM vitamins supplementation was not effective for porcine blastocyst development (Yoshioka et al., 2002). Likewise, the effect of MEM vitamins supplementation to *in vitro* embryo production is different for each animal species and the effect of MEM vitamins has not been clearly determined. Therefore, in this study, we researched the effects of MEM vitamins supplementation to *in vitro* maturation and culture medium on the development of *in vitro* porcine embryos in order to establish enhanced culture conditions.

MATERIALS AND METHODS

Chemicals and media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St, Louis, MO, USA). Solutions are expressed as percent dilutions (v:v) and all media used for *in vitro* maturation, *in vitro* fertilization, and i*n vitro* culture were pre-warmed to 39°C in a 5% CO_2 incubator with maximum humidity for 4 h before use.

Oocyte collection and *in vitro* maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 to 3 h in saline supplemented with 25 μg/ml gentamicin at 25-30°C. Cumulus oocyte complexes (COCs) were obtained by aspiration from follicles 2 to 6 mm in diameter using an 18-gauge needle connected to a 10-ml disposable syringe. Only COCs with compact cumulus cell layers and evenly granulated ooplasm were selected. The COCs were washed three times in HEPES-Tyrode-albumin-lactate-pyruvate medium (TALP medium) supplemented with 25 mM HEPES and 3 mg/ml BSA. Groups of 50 COCs were placed into 500 μl of BSA-free NCSU-23 solution with 0.57 mM cysteine, 10% porcine follicular fluid (pFF), 2.5 mM β-mercaptoethanol, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml human chorionic gonadotropin (hCG) and 10 IU/ml pregnant mare serum gonadotropin (PMSG) in each well of a 4-well multidish (Nunc, Roskilde, Denmark). After 22 h for maturation, oocytes were washed twice in the same maturation medium without PMSG and hCG and cultured in this medium for 22 h at 39°C in an atmosphere of 5% CO_2 and maximum humidity.

Supplementation of MEM vitamins on *in vitro* maturation medium

0.1, 0.2 and 0.4% of MEM vitamins (minimum essential medium vitamins, 11120-052, Life Technologies Inc., Grand Island, NY, USA) were added to *in vitro* maturation medium and *in vitro* culture medium as indicated. The blastocyte and hatched blastocyst development rate was then monitored following *in vitro* fertilization.

Sperm preparation and *in vitro* fertilization

Diluted porcine semen was produced by Dabby A.I. Center and stored at 17°C for 5 d. The semen was layered on top of a discontinuous Percoll density gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll) in a 15-ml centrifuge tube. The sample was centrifuged for 20 min at 500×g at room temperature. The spermatozoa collected in the bottom fraction were washed three times: twice in D- PBS containing 1 mg/ml BSA, 100 μg/ml penicillin and 75 μg/ml streptomycin at 500×g for 5 min, and once in modified Tris-buffered medium (mTBM). The spermatozoa were diluted with mTBM to give a final concentration of 3×10^6 spermatozoa/ml.

After the IVM period, oocytes were briefly treated with 0.1% hyaluronidase in Dulbecco's phosphate-buffered saline (D-PBS, Gibco, USA) supplemented with 1 mg/ml BSA to remove cumulus cells, and were washed 2 to 3 times with mTBM containing 1 mg/ml BSA and 2.5 mM caffeine sodium benzoate (Abeydeera et al., 1997). After washing, groups of 25 to 30 oocytes were placed in 48-μl droplets of mTBM in 60-mm petri dishes that had been covered with warm mineral oil. Two microliters of spermatozoa suspension was added to each fertilization drop, resulting in a final concentration of 2.5×10^5 spermatozoa/ml. Oocytes and spermatozoa were co-incubated for 6 h at 39°C and 5% CO_2 with maximum humidity.

In vitro culture

After sperm-oocyte co-incubation, presumptive zygotes (d 0) were washed 2 to 3 times in PZM3 supplemented with 3 mg/ml BSA. Thirty oocytes were transferred to 50 μl of PZM3 in a 60-mm petri dish, covered with warm mineral

oil, and cultured for 168 h at 39°C in a humidified atmosphere of 5% CO_2.

MEM vitamin supplementation on *in vitro* culture medium

The presumptive zygotes were cultured in medium containing 0.1, 0.2 and 0.4% of MEM vitamins as indicated. 0.4% MEM vitamins was supplemented on d 0, d 2 and d 4. After 48 h of *in vitro* culture, the fertilization rate was examined and the blastocyst and hatched blastocyst development rate was examined after 168 h.

Blastocyst differential staining

The zona pellucida of the blastocysts was removed with a 0.5% protease solution and washed 4 to 5 times in TL-HEPES solution with 0.1% PVA (TL-PVA). Zona-free blastocysts were incubated in a 1:5 dilution of rabbit anti-bovine whole serum in TL-PVA medium for 1 h. After being washed five more times in TL-PVA medium, blastocysts were re-incubated in a 1:10 dilution of a guinea pig complement in TL-PVA medium supplemented with 4 μg/ml propidium iodide (PI) and 4 μg/ml bisbenzimide for 1 h. The presumptive stained blastocysts were mounted on a slide and the cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan). The bisbenzimide-stained inner cell mass (ICM) nuclei fluoresced blue, and the trophectoderm (TE) nuclei, which were stained with both bisbenzimide and PI, fluoresced red or pink.

Statistical analysis

Data on the embryo development were analyzed by the x^2-test. All data for cell number were arcsine-transformed and analyzed by the General Linear Models Procedure with the Statistical Analysis System (SAS; Cary, USA). Treatment means were compared with Duncan's multiple range test; p-values less than 0.05 were considered statistically significant.

RESULTS

Altering the concentration of MEM vitamins on *in vitro* maturation medium has no effect on embryonic development rate

The concentration of supplemented MEM vitamins for *in vitro* maturation medium ranged from 0.1% to 0.4% in treated groups (Table 1). The *in vitro* fertilization rate was similar among all groups, ranging from 61.1% to 67.8%. Similarly, there was no significant difference among the treated groups on the embryonic development rate of the blastocyst which ranged from 11.4% to 14.3%. In addition the embryonic development rate of the hatched blastocyst ranged from 3.4% to 18.2%, which was also not significantly different among the treated groups.

Table 1. Effect of increasing concentration of MEM vitamins during *in vitro* maturation on the development of porcine oocytes

Concentration of MEM vitamins (%)	No. of examined oocytes	No. (%) of embryo developed to		
		≥2 cell	Blastocysts	Hatched blastocysts
Control[1]	210	132(62.9)	24(11.4)	3(12.5)
0.1	221	135(61.1)	27(12.2)	4(14.8)
0.2	215	140(65.1)	29(13.5)	1(3.4)
0.4	230	156(67.8)	33(14.3)	6(18.2)

[1] Control = NCSU23 without MEM vitamins.

Altering the concentration of MEM vitamins on *in vitro* culture medium has no effect on embryonic development rate

The concentration of supplemented MEM vitamins for *in vitro* culture medium ranged from 0.1% to 0.4% in treated groups (Table 2). The *in vitro* fertilization rate was not significantly different among the treated groups, ranging from 58.3% to 63.5%. Similarly, there was no significant difference among the treated groups on the embryonic development rate of the blastocyst which ranged from 4.3% to 14.4%. In addition, the embryonic development rate of the hatched blastocyst ranged from 7.7% to 31.3%, which was also not significantly different among the treated groups.

Altering the timing of addition of MEM vitamins supplementation to *in vitro* culture medium has no effect on embryonic development

The time to supplement of the MEM vitamins was experimentally altered from d 0 to d 4 in the treated groups (Table 3). The *in vitro* fertilization rate was not significantly different among the treated groups, ranging from 62.8% to 65.2%. Similarly, there was no significant difference among the treated groups on the embryonic development of the blastocyst which ranged from 11.5% to 13.6%. In addition, the embryonic development rate of the hatched blastocyst ranged from 7.1% to 26.7%, which was also not significantly different among the groups.

Table 2. Effect of increasing concentration of MEM vitamins during *in vitro* culture on the development of porcine *in vitro* fertilized embryos

Concentration of MEM vitamins (%)	No. of examined oocytes	No. (%) of embryo developed to		
		≥2 cell	Blastocysts	Hatched blastocysts
Control[1]	115	73(63.5)	15(13.0)	2(13.3)
0.1	125	76(60.8)	18(14.4)	4(22.2)
0.2	115	67(58.3)	13(11.3)	1(7.7)
0.4	130	78(60.0)	16(12.3)	5(31.3)

[1] Control = PZM3+0.3% BSA without MEM vitamins.

Table 3. Effect of altering the addition time of MEM vitamins during *in vitro* culture the on development of porcine *in vitro* fertilized embryos

Time (d)	No. of examined oocytes	No. (%) of embryo developed to		
		≥2 cell	Blastocysts	Hatched blastocysts
Control[1]	115	75(65.2)	15(13.0)	2(13.3)
d 0	110	70(63.6)	15(13.6)	4(26.7)
d 2	112	71(63.4)	14(12.5)	1(7.1)
d 4	113	71(62.8)	13(11.5)	1(7.7)

[1] Control = PZM3+0.3% BSA without MEM vitamins.

Altering the order of cultivation medium which contain MEM vitamins has no effect on embryonic development

To determine if addition of MEM vitamins to either or both *in vitro* media has an effect on embryonic development rate, four groups were grown under the following conditions: the double treated group in which 0.4% MEM vitamins supplementation was applied to both the *in vitro* maturation medium and the *in vitro* culture medium (+, +), one in which 0.4% MEM vitamins was supplemented only to the *in vitro* maturation medium (+, -), one in which 0.4% MEM vitamins was supplemented only to the *in vitro* culture medium (-, +), and the untreated group which had no MEM vitamins in any medium (-, -) (Table 4).

Within these various conditions, the *in vitro* fertilization rate ranged from 60.8% to 65.2%, which was not significantly different among the treated groups. The embryonic development of the blastocyst ranged from 12.0% to 15.1%, which was also not significantly different among the treated groups. Finally, the embryonic development rate of the breeding blastocyst ranged from 5.9% to 18.9%, which was also not significantly different among the treated groups.

MEM vitamins supplementation of *in vitro* maturation medium alone increases the total number of cells in the blastocyst

The total number of cells was 39.0±1.7 in the treated group (+, -) where MEM vitamins were supplemented to the *in vitro* maturation medium but not the *in vitro* culture medium (Table 5). This was significantly higher than 22.0±3.0 cells of the untreated group (-, -) and 29.5±8.6 cells of the double treated group (+, +) where both the *in vitro* maturation medium as well as the *in vitro* culture medium were supplemented with MEM vitamins ($p<0.05$). In addition, the number of trophectoderm (TE) cells was 34.7±1.5 in the treated group (+, -) with supplemented *in vitro* maturation medium and untreated *in vitro* culture medium, which was significantly higher than 18.3±2.1 of the untreated group (-, -) and 20.0±2.0 of the double treated group (+, +) ($p<0.05$) In contrast, the number of inner cell mass (ICM) cells among the total number of cells ranged from 3.7±1.5 to 4.3±1.1, which was not significantly different among the groups.

Table 4. Effect of addition of MEM vitamins during *in vitro* maturation (IVM)/*in vitro* culture (IVC) on the development of porcine oocytes

MEM vitamins (IVM/IVC)[1]	No. of examined oocytes	No.(%) of embryo developed to		
		≥2 cell	Blastocysts	Hatched blastocysts
(-/-)	250	163(65.2)	30(12.0)	2(6.7)
(+/-)	245	149(60.8)	37(15.1)	7(18.9)
(-/+)	255	163(63.9)	36(14.1)	6(16.7)
(+/+)	260	161(61.9)	34(13.1)	2(5.9)

[1] -: without, +: with.

DISCUSSION

The embryonic development rate of the blastocyst and hatched blastocyst in the group where 0.4% MEM vitamins were supplemented to the *in vitro* maturation medium but not the *in vitro* culture medium (+, -) was slightly higher than that of other treated groups, however it was not effective for significantly increasing embryo development. In contrast, supplementation of MEM vitamins to the *in vitro* maturation medium but not the *in vitro* culture medium (+, -) resulted in a significant increase in the total numbers of cells compared to the untreated group (-, -) or the double treated group (+, +) ($p<0.05$).

There have been many attempts to create an *in vitro* culture condition for oocytes which mimic the natural environment of the internal mammalian condition. As results of these studies, several effective improvements have been made such as a method to decrease the oxygen concentration in the *in vitro* culture medium (Fukui et al., 1992), supplementation of an alternative energy source (Choi et al., 2002; Rizos et al., 2003) growth factor addition (Saito et al., 1984; Ogawa et al., 1987), supplementation of antioxidant that protects *in vitro* oocyte from free radicals (Murray et al., 1990; Ali et al., 2003), and supplementation of serum (Nagai et al., 1988; Mattioli et al., 1989).

Table 5. Effect of MEM vitamins supplements on cell number of porcine blastocysts developed from *in vitro* produced embryos

MEM viamins (IVM/IVC)[1]	No. of examined blastocysts	No.(%) of cells (mean±SEM)		
		ICM	TE	Total
(-/-)	13	3.7±2.1	18.3±2.1[a]	22.0±3.0[a]
(+/-)	12	4.3±1.1	34.7±1.5[b]	39.0±1.7[b]
(-/+)	14	3.7±2.1	29.7±9.3[ab]	33.3±9.9[ab]
(+/+)	13	3.7±1.5	20.0±2.0[a]	29.5±8.6[a]

[a, b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

[1] -: without, +: with.

Additionally, in the case of bovine, embryo development was improved when it was co-cultured with mouse fetus fibroblast, by producing either an unknown growth-promoting factor or by removing a risk factor for oocytes that exist in the medium from the coculture cell (Eyston and First, 1989). However, currently developed *in vitro* environment was not yet appropriate for oocyte culture.

Although Yoshioka et al. (2002) reported that MEM vitamins was not effective in porcine embryo development, Rabbit (Kand and Bavister, 1988) and hamster (Mishra and Seshagiri, 1998) were effective in improving the embryo development rate. Likewise, there is still controversy regarding the effect of supplementation of water-soluble vitamins on the *in vitro* development of embryos of mammalian animals.

The kinds of vitamins that are supplemented with MEM vitamins generally consist of myo-inositol (0.02%), nicotinamide (0.01%), pantothenic acid (0.01%) which is the complex of vitamin B, pyridoxine hydrochloride (B6, 0.01%), riboflavin (0.001%), thamine HCl (0.01%), choline chloride (0.01%) and folic acid (0.01%) (Hong et al., 2004). It was reported that these vitamins are imported actively or passively into the cell and prevent the loss of oocyte viability that occurs in suboptimal culture conditions. Although the overall transfer system as well as the mutual cooperation of these vitamins are still unknown, the roles of individual vitamins have been identified. For example, choline plays an important role in maintaining the metabolism of phospholipids and cell structure (McDowell, 2000), folic acid is important for proteolysis, DNA synthesis and in energy production (McDowell, 2000), nicotinamide is important for glycolysis and the redox reactions of cells including most of the citric acid cycle during cell respiration (Stryer, 1988), riboflavin plays a role as a precursor for two important subsidiary enzymes which are important for energy production and flavin mononucleotide as well as flavin adenine dinucleotide act as hydrogen carriers to produce energy in the form of ATP through the metabolism of lipids and carbohydrates (McDowell, 2000).

Also, regarding the embryo, pantothenate promotes the development of the blastocyst and is important for embryo vitality, at least in the hamster (McKiernen and Bavister, 2000). Inositol, which is known to play an important role in the *in vitro* maturation of oocyte, is involved in phospholipid synthesis for new cell membranes (Hawthorne, 1982; Vance, 1986), pronucleus formation during *in vitro* fertilization (Kane and Bavister, 1988), and it acts as the phosphatidylinositol second messenger (Kane and Bavister, 1988).

These processes are responsible for mediating numerous hormone and growth factor reactions. When these vitamins are supplemented to *in vitro* maturation medium, they have the potential to improve the effect of PMSG, HCG and EGF on the *in vitro* maturation medium. Also, it is known that vitamin B6 displays antioxidant activity that inhibits free radicals (Olson and Seidel, 2000) during the *in vitro* culture of embryo (Kamat and Devasagayam, 1996; Mcdowell, 2000; Kitagawa et al., 2004). In addition, Lane and Gardner (1998) reported that vitamins play roles in inhibiting the vitality loss and unstable metabolism due to inappropriate culture conditions.

In conclusion, the results of this study demonstrate that MEM vitamins supplementation to *in vitro* maturation medium alone does have an effect on embryo development as seen by an increase in cell numbers. Therefore, the effect of MEM vitamins can be offset by hormones, albumin or other components that are supplemented to *in vitro* maturation medium. However, the supplementation of MEM vitamins to *in vitro* culture medium did not affect development of the porcine embryo *in vitro*. Further studies are required to understand the reason for the suppression of porcine embryos produced *in vitro* with supplementation of MEM vitamins to the culture medium.

REFERENCES

Abeydeera, L. R. and B. N. Day. 1997. Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol. Reprod. 57:729-734.

Abeydeera, L. R., W. H. Wang, T. C. Cantley, A. Rieke, C. N. Murphy, R. S. Prather and B. N. Day. 2000. Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. Theriogenology 54:787-797.

Abeydeera, L. R., W. H. Wang, R. S. Prather and B. N. Day. 2001. Effect of incubation temperature on vitro maturation of porcine oocytes: Nuclear maturation, fertilization and developmental competence. Zygote 9:331-337.

Ali, A. A., J. F. Bilodeau and M. A. Sirard. 2003. Antioxidant requirements for bovine oocytes varies during *in vitro* maturation, fertilization and development. Theriogenology 59:939-949.

Camous, S., Y. Heyman, W. Menezo and Y. Menezo. 1984. Cleavage beyong the block stage and survival after transfer of early bovine embyos cultured with trophoblastic vesicles. J. Reprod. Fertil. 72:479-485.

Chang, H. S., W. T. Cheng, H. K. Wu and K. B. Choo. 2000. Identification of genes expressed in the epithelium of porcine oviduct containing early embryos at various stages of development. Mol. Reprod. Dev. 56:331-335.

Choi, Y. H., B. C. Lee, J. M. Lim, S. K. Kang and W. S. Hwang. 2002. Optimization of culture medium for cloned bovine embryos and its influence on pregnancy and delivery outcome. Theriogenology 58:1187-1197.

Dobrinsky, J. F., L. A. Johnson and D. Rath. 1996. Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. Biol. Reprod. 55:1069-1074.

Eyestone, W. H. and N. L. First. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil. 85:710-720.

Fukui, Y., Y. Terawaki and H. Ono. 1992. Effect of gonadotropins and steroids and culture on bovine oocytes maturation. Theriogenology 18:161-175.

Hawthorne, J. N. 1982. Inositol phospholipids. In: Phospholipids (Ed. J. N. Hawthorne and G. B. Ansell). Amsterdam: Elsevier Biomedical Press. 263-278.

Heyman, Y., Y. Menezo, P. Chesne, S. Camous and V. Garnier. 1987. *In vitro* cleavage of bovine and ovine early embryos: Improved development using co-culture with trophoblastic vesicles. Theriogenology 27:59-68.

Hong, J. Y., H. Y. Yong, B. C. Lee, W. S. Hwang, J. M. Lim and S. Lee. 2004. Effects of amino acid on maturation, fertilization and embryo development of pig follicular oocytes in two IVM media. Theriogenology 62:1473-1482.

Iritani, A., K. Niwa and H. Imai. 1978. Sperm peneration of pig follicular oocytes matured in culture. J. Reprod. Fertil. 54:379-383.

Kamat, J. P. and T. P. Devasagayam. 1996. Methylene blue plus light-induced lipid peroxidation in rat liver microsomes: inhibition by nicotinamide (Vitamin B3) and other antioxidants. Chem. Biol. Interact. 99:1-16.

Kane, M. T. 1988. The effects of water-soluble vitamins on the expansion of rabbit blastocysts *in vitro*. J. Exp. Zool. 245:220-223.

Kane, M. T. and B. D. Bavister. 1988. Vitamins requirements for development of eight-cell hamster embryos to hatching blastocysts *in vitro*. Biol. Reprod. 39:1137-1143.

KiKuchi, K., A. Onishi, N. Kashiwazaki, M. Iwamoto, J. Noguchi, H. Kaneko, T. Akita and T. Nagai. 2002. Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. Biol. Reprod. 66:1033-1041.

Kitagawa, Y., K. Suzuki, A. Yoneda and T. Watanabe. 2004. Effects of oxygen concentration and antioxidants on the *in vitro* developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 62:1186-1197.

Krishnaswamy, K. and K. J. Sushil. 2004. Effect of vitamin B on oxygen radicals, mitochondrial membrane potential, and lipid peroxidation in H_2O_2-treated U937 monocytes. Free Radic. Biol. Med. 36:423-428.

Lane, M. and D. K. Gardner. 1998. Amino acids and vitamins prevent culture-induced metabolic perturbation and associated loss of viability of mouse blastocysts. Hum. Reprod. 13:991-997.

Legge, M. and M. H. Sellens. 1991. Free radical sacvengers ameliorate the 2-cell block in mouse embryo culture. Hum. Reprod. 6:867-871.

Lim, J. M., B. C. Reggio, R. A. Godke and W. Hansel. 1997. Perifusion culture system for bovine embryos: improvement of embryo development by use of bovine oviduct epithelial cells, an antioxidant and polyvinyl alcohol. Reprod. Fertil. Dev. 94:411-418.

Mattioli, M., M. L. Bacci, G. Geleati and E. Seren. 1989. Developemental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology 31:1201-1207.

May, J. V., J. P. Frost and D. W. Schomberg. 1988. Differential effects of epidermal growth factor, somatomedian-C/insulin-like growth factor and transforming growth factor-beta on porcine granulose cell DNA synthesis and cell proliferation. Endocrinology 123:168-179.

McKiernen, S. H. and B. D. Bavister. 2000. Culture of one-cell hamster embryos with wate soluble vitamins: pantothenate stimulates blastocyst production. Hum. Reprod. 15:157-164.

McDowell, L. R. 2000. Vitamins in animal and human nutrition. Ames. IA, Iowa State University Press.

Mishra, A. and P. B. Seshagiri. 1998. Successful developoment *in vitro* of hamster 8-cell embryos to 'zona-escaped' and attached blastocysts: assessment of qulaity and trophoblast outgrowth. Reprod. Fertil. Dev. 10:413-420.

Motlik, J., N. Grozet and J. Fulka. 1984. Meiotic competence *in vitro* of pig oocytes isolated from early antral follicles. J. Reprod. Fertil. 72:323-328.

Murray, R. K., D. K. Granner, P. A. Mayer and V. W. Rodwell. 1990. Lipid peroxidation is a source of free radicals *in vitro*. Harper's Biochem. 20:142-143.

Nagai, T., T. Takahashi, H. Masuda, Y. Shioya, M. Kuwayama, M. Fukushima, S. Iwasaki and A. Hanade. 1988. *In vitro* fertilization of pig oocytes by frozen boar spermatozoa. J. Reprod. Fertil. 84:585-591.

Ogawa, T., R. P. Ono and T. Marrss. 1987. The effect of serum fractions on single-cell mouse embryos *in vitro*. J. *In vitro* Fertil. Embryo. Transfer. 4:153-158.

Olson, S. E. and Jr. G. E. Seidel. 2000. Culture of *in vitro*-produced bovine embryos with vitamin E improves development *in vitro* and after transfer to recipients. Biol. Reprod. 62:248-252.

Prather, R. and B. N. Day. 1998. Practical consideration for the *in vitro* production of pig embryos. Theriogenology 49:23-32.

Petters, R. M. and K. D. Wells. 1993. Culture of pig embryos. J. Reprod. Fertil. Suppl. 48:61-73.

Rizos, D. A., S. Glutierrez-Adan, J. Perez-Garnels, M. De La Fuente, P. P. Boland and P. Lonergan. 2003. Bovine embryos culture in the presence or absence of serum implication for blastocyst development, cryotolerance, and messenger RNA expression. Biol. Reprod. 68:236-243.

Saito, H., T. Berger, D. R. Mishell Jr and R. P. Marrs. 1984. The effect of serum fractions on embryo growth. Fertil. Steril. 41:761-765.

Stokes, P. J., L. R. Abeydeera and H. J. Leese. 2005. Development of porcine embryos *in vivo* and *in vitro*; evidence for embryo 'cross talk' *in vitro*. Dev. Biol. 284:62-71.

Stryer, L. 1988. Biochemistry. New York: W. H. Freeman. 322-325.

Vance, D. E. 1986. Phospholipid metabolism in eukaryotes. In: Biochemistry of lipids and membranes (Ed. J. E. Vance). Menlo Park, CA: The Benjamin/Cummings Publishing Co. Inc. 242-270.

Wang, W. H., M. Hosoe and Y. Shioya. 1997. Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. J. Reprod. Fertil. 109:247-255.

Xu, X. J., Q. Z. Yang, R. C. Qian and Z. Nakahara. 1992. Development of *in vitro* fertilized bovine ova in two co-culture systems. Anim. Reprod. 1:372-374.

Yoshioka, K., C. Suzuki, A. Tanaka, I. M. Anas and S. Iwamura. 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66:112-119.