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Effect of Essential and Nonessential Amino Acids in North Carolina State University (NCSU)-23 Medium on Development of Porcine *In vitro* Fertilized Embryos

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ABSTRACT: The present study was conducted to examine the effect of different levels of essential and nonessential amino acid in NCSU-23 medium on the *in vitro*-produced porcine embryo as it develops from the zygote to the blastocyst stage. Four experiments were performed, each with a completely randomized design involving 5 to 8 replications of treatments. In order to know the effect of nonessential amino acids in NCSU-23 medium, 0, 5, 10 and 20 μl/ml MEM were supplemented there to, (Exp. 1) and the medium was supplemented with same level of essential amino acids (Exp. 2). The combined effect of nonessential (0, 5, 10 and 20 μl/ml MEM) and essential amino acids (0, 5, 10 and 10 μl/ml MEM) in NCSU-23 medium (Exp. 3), first 72 h with non-essential amino acids (at 0, 5, 10 and 20 μl/ml MEM), and last 4 d with essential amino acids with the same level as NEAA (Exp. 4) were examined. The embryo development was monitored and the quality of blastocysts was evaluated by counting the number of total cells and determining the ratio of inner cell mass (ICM) to trophoectoderm (TE) cells. When Eagle's nonessential amino acids (MEM) added to NCSU-23 medium, it significantly increased the likelihood of development to the 2- to 4-cell stage and subsequent blastocyst development. Supplementation of different levels of essential amino acids in the NCSU-23 medium decreased cleavage rate, rate of morula and blastocyst development and the number of ICMs. In the case of the combined effect of essential and nonessential amino acids, better and significant results were found for blastocysts, hatching blastocysts and for ICM numbers which were also dose dependent. With respect to the biphasic effect of nonessential and essential amino acids, nonessential amino acids increased cleavage whereas essential amino acids increased the total cell number. Neither the nonessential nor the essential group of amino acids, on their own, affected blastocyst cell number or the differentiation of cells in the blastocyst. In conclusion, this study determined the role of nonessential and essential amino acids in the culture of the porcine embryo and showed that the embryo requires different levels of amino acids as it develops from the zygote to the blastocyst stage. (**Key Words** : NCSU-23, Essential, Nonessential, Amino Acids, Porcine, IVF)

INTRODUCTION

Amino acids serve a variety of physiological functions, including: the synthesis of proteins and nucleotides (Epstein and Smith. 1973; Alexiou and Leese, 1992; Katchadourian et al., 1994), nutrition and energy provision (Lane and Gardner 1997a; 1998; Gardner, 1998; Houghton et al., 2002), osmoregulation (Van Winkle and Campione, 1996; Dumoulin et al., 1997; Dawson et al., 1998), protection against oxidative stress (Lindenbaum, 1973; Nasr-Esfahani et al., 1992; Jang et al., 2005), pH regulation (Bavister and McKiernan, 1993; Edwards et al., 1998), signaling molecule biosynthesis (Wu and Morris, 1998), trophectoderm differentiation (Martin and Sutherland, 2001) and basement membrane formation between primitive endoderm and ectoderm (Biggers et al., 2000).

The uterine endometrium synthesizes and secretes a variety of proteins to nourish the early conceptus in pigs (Ka and Bazer, 2005). It has also been suggested that *in vitro* produced embryos should be exposed to amino acids as early as the oocyte stage, as this increases oocyte maternal mRNA levels and promotes preimplantation development (Watson et al., 2000). In support of this opinion, it is notable that a brief exposure of zygotes to

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amino acid-free conditions depresses their developmental capacity and blastocyst cell numbers (Gardner and Lane, 1996).

Technologies on preimplantation porcine embryos have been developed quickly and significantly (Niwa and Funahashi, 1999). A major step toward improving media for the culture of bovine embryos was the discovery that the addition of Eagle's amino acids improved embryo development (Takahashi and First, 1992; Kim et al., 1993; Gardner, 1994; Rosenkrans and First; 1994). Much of our understanding of the way that amino acids affect mammalian embryo development and subsequent viability has come from studies on the hamster (Carney and Bavister, 1987; Bavister and Arlotto, 1990; Bavister and Mckiernan, 1993; Mckiernan et al., 1995), mouse (Mehta and Kiessling, 1990; Gardner and Lane, 1993; Lane and Gardner, 1994; Gardner and Lane, 1996; Lane and Gardner, 1997; Lane and Gardner, 1997a), and rat (Zhang and Armstrong, 1990). These studies have determined that amino acids can be either stimulatory or inhibitory to embryo development *in vitro* and that the presence of amino acids in culture media has a significant effect on the viability of embryos and postimplantation development.

The supplementation of amino acids to a culture medium significantly improved the development of hamster (Schini and Bavister, 1988), mouse (Lane and Gardner, 1994; Lamb and Leese, 1994; Lane and Gardner, 1997), and cow embryos (Rosenkrans and First, 1994; Bavister and Arlotto, 1990; Bavister and Mckiernan, 1993).

Subsequent studies have revealed a biphasic requirement for amino acids during the preimplantation period (Lane and Gardner, 1997a; Steeves and Gardner, 1999). The zygote and cleavage-stage embryo benefit from the inclusion of Eagle's nonessential amino acids and glutamine. Significantly, this group of amino acids bears a striking homology to those present at high levels in the female reproductive tract. By contrast, after the eight-cell stage the mammalian embryo benefits from the presence of a more complex array of amino acids (Steeves and Gardner, 1999), with Eagle's essential amino acids being found to stimulate the development of the inner cell mass (ICM) (Lane and Gardner, 1997a; Lane et al., 2001). Significantly, equivalent rates of implantation to *in vivo*-developed blastocysts were obtained when mouse zygotes were cultured with nonessential amino acids up to the eight-cell stage, followed by culture to the blastocyst stage in the presence of 20 amino acids (Lane and Gardner, 1997a).

The present study was undertaken to examine the effect of essential and nonessential amino acids alone or in combination, as well as the biphasic effect in NCSU-23 medium on the development of porcine IVF embryos.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise stated.

Oocyte collection and *in vitro* maturation

Ovaries were retrieved from prepubertal gilts at a local abattoir and were transported to the laboratory in 0.9% (wt/vol) NaCl solution at 30 to 35°C within 2 h from collection. Follicular fluid and cumulus-oocytes complexes (COCs) from follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. Compact COCs were selected and cultured in tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA) supplemented with 10 ng/ml epidermal growth factor, 4 IU/ml of equine chorionic gonadotropin (eCG; Intervet, Boxmeer, Netherlands), 4 IU/ml of human chorionic gonadotropin (hCG; Intervet), 10% (v/v) porcine follicular fluid (pFF). The pFF was aspirated from 3 to 7 mm sized follicles, from the prepubertal gilt ovaries. After centrifugation at 3,000×g for 30 min, supernatants were collected and filtered sequentially through 1.2 and 0.45 M syringe filters (Gelman Sciences, Ann Arbor, MI) and were heat inactivated at 56°C for 30 minutes. Prepared pFF was then stored at -20°C until use. The COCs, surrounded by a compact cumulus mass and with evenly granulated cytoplasm, were selected and washed 3 times in oocyte maturation medium containing hormone supplements. A group of 50 to 60 oocytes were transferred into each well of a Nunc 4-well multidish (Nunc, Roskilde, Denmark) containing 500 μl of culture medium and equilibrated in a 5% CO_2 incubator. After 20 to 22 h of maturation culturing, oocytes were washed 3 times in the maturation medium without hormone supplements and transferred into 500 μl drops of the same medium for an additional 20 to 22 h of culture.

In vitro oocyte fertilization and embryo culture

Frozen porcine semen was thawed at 39°C for 1 min in a water bath, diluted in 10 ml Dulbecco's PBS (*Invitrogen*) supplemented with 0.1% BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate and centrifuged twice at 350×g, for 2 min each time. The sperm pellet was resuspended in modified Tris-buffered medium (mTBM) containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate and 0.1% (w/v) BSA. Groups of 25 matured oocytes were placed into 40 μl mTBM droplets and inseminated with 2×10^6 spermatozoa/ml for 6 h at 39°C. Embryos were cultured in NCSU-23 medium (Petters and Wells, 1993). Essential and non-essential amino acids were added to NCSU-23 medium as per experimental design and

Table 1. Effect of different levels of nonessential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the development of *in vitro* fertilized porcine embryos

Level of NEAA	Embryo development (%), N (Mean±SD)					
(μl/ml)	N	2-4 cell	8-16 cell	Morula	Blastocysts	Hatched blastocysts
0	347	259 (72.4±8.5)^{b}	159 (45.7±6.6)	110 (31.0±7.1)	56 (15.7±2.0)^{b}	4 (1.0±1.6)^{b}
5	324	287 (82.3±9.2)^{a}	188 (56.0±8.9)	124 (37.6±8.1)	70 (21.9±6.6)^{a}	16 (5.0±4.3)^{a}
10	353	303 (85.3±3.9)^{a}	182 (51.2±6.7)	121 (33.1±6.6)	84 (23.4±6.2)^{a}	26 (8.2±5.3)^{a}
20	363	312 (85.0±5.7)^{a}	190 (51.7±12.1)	128 (36.4±11.7)	82 (23.2±7.4)^{a}	18 (4.7±3.2)^{ab}

N = Number of oocytes. SD = Standard deviation.
Data were obtained after eight replications.
^{a,b} Within the same column, values with different superscripts were significantly different ($p<0.05$).

Table 2. Effect of nonessential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the number of inner cell masses (ICM), trophectoderms (TE) and total cell number of porcine *in vitro* fertilized embryos

Level of NEAA	Number of cells (Mean±SD)*			
(μl/ml)	ICM	TE	Total	TE/ICM
0	16.5±4.1	39.4±10.0^{b}	58.6±15.0	2.5±0.8
5	19.3±4.7	63.0±15.7^{a}	81.8±21.4	3.9±1.4
10	17.0±6.3	48.8±22.3^{ab}	68.3±22.7	3.2±1.9
20	16.0±4.2	45.0±19.8^{ab}	61.0±19.8	3.1±1.7

* Values shown are obtained with 15 embryos from three replicates.
^{a,b} Within the same column, values with different superscripts were significantly different ($p<0.05$).

diluted with embryo-tested water and then filter-sterilized (0.2 μm Millipore filters). Osmolarity measured by a freezing point depression osmometer (Osmomat 030, Gonotec GmbH) was 280±5 for NCSU-23. Embryos were cultured in NCSU-23 medium supplemented with or without essential and nonessential AA which was covered with mineral oil, in 5% O_2, 5% CO_2, and 90% N_2 at 39°C for 7 days.

Embryo evaluation and total cell count

Throughout *in vitro* embryo culture, the progression of developmental stages and morphology was observed using an inverted microscope (Zeiss Axiovert 35 M) and recorded daily. Embryos in each group were taken out of the incubator for counting and evaluation for a period of 5 min. Two to 4-cell, 8- to 16-cell, morula and blastocyst stages were distinguished. At the end of the embryo culture (at 7 days), total cell numbers per blastocyst were determined using Hoechst 33342 (B-2261) fluorescent DNA staining technique (Bagis et al., 2002; Bagis and Odaman, 2004). The quality of each blastocyst was assessed by differential staining of the ICM and the TE cells according to the modified staining procedure of Thouas et al. (2001). Briefly, hatched blastocysts were used as such and non-hatched blastocysts were treated with 0.25% (wt/vol) pronase for 5 min to dissolve the zonae pellucidae. After rinsing with NCSU-23 washing medium, zona free blastocysts were stained with 0.01% (wt/vol) bisbenzimide for 1 h. After rinsing with NCSU-23 washing medium, blastocysts were treated with 0.04% (v:v) Triton X-100 for 3 min followed by treatment with 0.005% (wt/vol) propidium iodide for 10 min. After rinsing with NCSU-23 washing medium, stained blastocysts were mounted on glass slides under a cover slip and examined under an inverted microscope (Nikon Corp.) equipped with epifluorescence. The ICM nuclei labeled with bisbenzimide appeared blue and TE cell nuclei labeled with both bisbenzimide and propidium iodide appeared pink. Any blastocysts without dual stain and/or with less than 20 total cell nuclei were excluded from data analysis.

Experimental design

Four experiments were performed, each with a completely randomized design involving 5 to 8 replications of treatments. In order to know the effect of nonessential amino acids in NCSU-23 medium, 0, 5, 10 and 20 μl/ml MEM were supplemented there to, (Exp. 1) and the medium was supplemented with same level of essential amino acids (Exp. 2). The combined effect of nonessential (0, 5, 10 and 20 μl/ml MEM) and essential amino acids (0, 5, 10 and 10 μl/ml MEM) in NCSU-23 medium (Exp. 3), first 72 h with non-essential amino acids (at 0, 5, 10 and 20 μl/ml MEM), and last 4 d with essential amino acids with the same level as NEAA (Exp. 4) were examined. The embryo development was monitored and the quality of blastocysts was evaluated by counting the total number of cells and determining the ratio of ICM to TE cells.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and protected least significant difference (LSD) test using the SAS program to determine differences among experimental groups. When a significant treatment effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was considered existent where the *P* value was less than 0.05.

RESULTS

Effect of nonessential amino acids (Experiment I)

The effect of different levels of NEAA in NCSU-23

Table 3. Effect of different levels of essential amino acids (EAA) in North Carolina State University (NCSU)-23 medium on the development of *in vitro* fertilized porcine embryos

Level of EAA	Embryo Development (%), N (Mean±SD)					
(μl/ml)	N	2-4 cell	8-16 cell	Morula	Blastocysts	Hatched blastocysts
0	234	202 (85.9±8.9)^{a}	144 (60.6±13.5)^{a}	84 (35.5±8.4)^{a}	37 (15.9±1.1)^{ab}	1 (0.4±0.9)
5	235	190 (81.6±8.0)^{a}	135 (57.3±8.0)^{a}	67 (28.9±8.1)^{a}	44 (18.9±3.7)^{a}	3 (1.3±3.0)
10	240	175 (73.9±11.1)^{ab}	95 (37.9±13.6)^{b}	43 (17.5±3.0)^{b}	30 (12.4±4.3)^{bc}	1 (0.4±1.0)
20	238	154 (65.5±11.7)^{b}	65 (25.7±10.8)^{b}	33 (13.3±4.3)^{b}	27 (11.1±3.6)^{c}	1 (0.5±1.0)

N = Number of oocytes. SD = Standard deviation.
Data were obtained after eight replications.
$^{a, b}$ Within the same column, values with different superscripts were significantly different ($p<0.05$).

Table 4. Effect of essential amino acids (EAA) in North Carolina State University (NCSU)-23 medium on the number of inner cell masses (ICM), trophectoderms (TE) and total number of cells of porcine *in vitro* fertilized embryos

Level of EAA	Number of cells (Mean±SD)*			
(μl/ml)	ICM	TE	Total	TE/ICM
0	14.8±6.3^{a}	26.8±8.5	41.6±9.8	2.2±1.4
5	9.4±2.2^{b}	34.2±13.7	43.6±13.2	3.9±2.0
10	9.2±1.9^{b}	27.4±13.5	36.6±13.7	3.1±1.6
20	12.4±2.5^{ab}	24.8±10.0	37.2±10.8	2.0±0.9

* Values shown are obtained with 15 embryos from three replicates.
$^{a, b}$ Within the same column, values with different superscripts were significantly different ($p<0.05$).

medium on the development of porcine embryos and on cell allocation in blastocysts of *in vitro* fertilized porcine embryos is shown in Table 1 and 2. In Table 1, it is shown that cleavage as 2-4 cell, blastocysts and hatched blastocysts differ significantly ($p<0.05$) from control group. All treated groups (5, 10, and 20 μl/ml NEAA) were equally effective but significantly better than control group at cleavage and blastocysts stages. The percentages of cleavage at control, 5, 10 and 20 μl/ml NEAA were 72.4, 82.3, 85.3 and 85.5%, respectively. The percentages of blastocysts at control, 5, 10 and 20 μl/ml NEAA were 15.7, 21.9, 23.4 and 23.2%, respectively. However, in the case of hatching blastocysts, all treated groups were equally effective, and 20 μl/ml NEAA were also equally effective with control group. The percentages of hatching blastocysts at control, 5, 10 and 20 μl/ml NEAA were 1.0, 5.0, 8.2 and 4.7%, respectively. In Table 2, no statistically significant differences in ICM, total cell numbers and TE/ICM were observed in blastocysts obtained from the control as well as treated groups. In contrast, TE cells in blastocysts differ significantly ($p<0.05$) between control and treated groups. All treated groups were equally effective whereas control, 10 and 20 μl/ml NEAA were equally effective in the case of TE numbers. The average number of TE at control, 5, 10 and 20μl/ml NEAA was 39.4, 63.0, 48.8 and 45.0, respectively (Table 2).

Effect of essential amino acids (Experiment II)

The effect of different levels of EAA in NCSU-23 medium on the development of embryos and on cell allocation in blastocysts of *in vitro* fertilized porcine embryos is shown in Table 3 and 4. As shown in Table 3, there were significant ($p<0.05$) differences at cleavage, 8-16 cell, morula and blastocyst stages between control and treated groups. At cleavage stage, control, 5 and 10 μl/ml EAA were equally effective and 10 and 20 μl/ml EAA were also equal in their effect. The percentages of cleavage decreased as the level of EAA was increased. The percentages of cleavage at control, 5, 10 and 20 μl/ml EAA were 85.9, 81.6, 73.9 and 65.5%, respectively. At 8-16 cell and morula stage, control and 5 μl/ml EAA were equally effective and 10 and 20 μl/ml EAA were also equal in their effect. The percentage of 8-16 cell and morula decreased as the level of EAA was increased. The percentages of 8-16 cell and morula at control, 5, 10 and 20 μl/ml EAA were 60.6 vs. 35.5, 57.3 vs. 28.9, 37.9 vs. 17.5 and 25.7 vs. 13.3%, respectively. At the blastocyst stage, control and 5 μl/ml EAA were equally effective and control also had an effect equal to that of 10 μl/ml EAA. On the contrary, 10 and 20 μl/ml EAA were also equally effective. The percentages of blastocysts at control, 5, 10 and 20 μl/ml EAA were 15.9, 18.9, 12.4 and 11.1%, respectively. However, ICM numbers were also equal at control and 20 μl/ml EAA and all treated groups were equally effective in terms of ICM numbers in blastocysts.

Combined effects of non-essential and essential amino acids (Experiment III)

The combined effect of different levels of essential and nonessential amino acids in NCSU-23 medium on the development and cell allocation in blastocysts of porcine IVF embryos is shown in Table 5 and 6. As shown in Table 5, treated groups differ significantly ($p<0.05$) from the control group in the cases of 8-16 cell, morula, blastocysts and hatched blastocysts stages of embryo development. At 8-16 cell, morula, blastocyst and hatched blastocyst stages, treated groups were equally effective. However, at blastocyst and hatched blastocyst stages, control, 5 EAA+ 10 NEAA μl/ml and 10 EAA+10 NEAA μl/ml were equally effective. On the contrary, all treated groups were equally

Table 5. Combined effect of different levels of essential amino acids (EAA) and non-essential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the development of *in vitro* fertilized porcine embryos

Level of EAA and NEAA (μl/ml)		Embryo Development (%), N (Mean±SD)					
EAA	NEAA	N	2-4 cell	8-16 cell	Morula	Blastocysts	Hatched blastocysts
0	0	239	198 (82.8±7.8)	136 (56.9±9.1)^{a}	101 (42.3±5.6)^{a}	39 (16.3±2.6)^{b}	4 (1.7±1.7)^{b}
5	10	223	167 (74.5±10.6)	101 (45.2±5.1)^{b}	73 (32.9±3.9)^{b}	43 (19.3±2.9)^{ab}	11 (5.1±3.1)^{ab}
10	10	206	168 (81.9±8.3)	86 (42.2±8.3)^{b}	62 (30.6±6.4)^{b}	38 (18.9±6.1)^{ab}	13 (6.3±2.5)^{ab}
20	10	212	177 (83.5±4.7)	77 (36.2±9.4)^{b}	58 (27.2±7.2)^{b}	49 (22.9±6.2)^{a}	19 (8.8±5.6)^{a}

N = Number of oocytes. SD = Standard deviation.
Data were obtained after eight replications.
^{a,b} Within the same column, values with different superscripts were significantly different ($p<0.05$).

Table 6. Combined effects of different levels of essential amino acids (EAA) and non-essential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the number of inner cell masses (ICM), trophectoderms (TE) and total number of cells of porcine *in vitro* fertilized embryos

Level of EAA and NEAA (μl/ml)		Number of cells (Mean±SD)*			
EAA	NEAA	ICM	TE	Total	TE/ICM
0	0	14.7±2.1^{b}	38.2±8.4	54.5±8.1	2.6±0.7
5	10	18.5±3.9^{ab}	48.0±12.1	63.2±8.9	2.6±0.6
10	10	18.2±6.6^{ab}	48.0±18.8	66.2±20.9	2.8±1.3
20	10	23.3±4.0^{a}	48.8±11.3	61.8±1.6	2.1±0.3

* Values shown are obtained with 15 embryos from three replicates.
^{a,b} Within the same column, values with different superscripts were significantly different ($p<0.05$).

effective in terms of their ICM values, and the control was also equally effective in terms of ICM numbers for 5 EAA+ 10 NEAA μl/ml and 10 EAA+10 NEAA μl/ml treated groups (Table 6).

Biphasic effect of non-essential and essential amino acids (Experiment IV)

Biphasic effect of nonessential and essential amino acids in NCSU-23 medium on the development and cell allocation in blastocysts of porcine IVF embryos is shown in Table 7 and 8. As shown in Table 7, there were no significant differences between control and treated groups from cleavage to hatched blastocyst stages of embryo development. However, from Table 7 it appears that only in the case of total cell numbers, there were significant differences in treated and control groups ($p<0.05$). All treated groups were equally effective whereas control and 10 EAA+10 NEAA μl/ml were also equally effective.

DISCUSSION

The present study was conducted to examine the effect of different levels of essential and nonessential amino acid in NCSU-23 medium of the *in vitro*-produced porcine embryo as it develops from the zygote to the blastocyst. When Eagle's nonessential amino acids (MEM) added to NCSU-23 medium, it significantly increased development to the 2- to 4-cell stage and subsequent blastocyst development. This is in agreement with the findings of Steeves and Gardner (1999) where they found a similar pattern of development in bovine cells. Previous studies have illustrated the stimulatory effects of the combination of glutamine and the nonessential amino acids in the development of mammalian embryos *in vitro*. Culture of mouse embryos from the 2-cell stage with Eagle's MEM nonessential amino acids and glutamine significantly increased blastocyst formation, cell number, hatching rate, and postimplantation development (Gardner and Lane, 1993; Lane and Gardner, 1994). The nonessential amino acids and glutamine were found to stimulate the development of the mouse embryo by decreasing the time of the first three cleavage divisions (Lane and Gardner, 1997). When added during the entire culture period, the nonessential amino acids and glutamine have also been shown to stimulate the development of the ruminant embryo (Gardner and Lane, 1994; Liu and Foote, 1995). The higher cell numbers, as TE and total cell observed in the blastocysts cultured with different levels of nonessential amino acids in the present study, might reflect a higher embryo viability, although this needs to be confirmed through embryo transfer.

The amino acids classified as nonessential for somatic cells by Eagle (Eagle, 1959) have been shown to stimulate development of the cleavage stage embryo as well as stimulating blastocoel formation in the postcompaction stage embryo (Gardner and Lane, 1993; Lane and Gardner, 1997). Interestingly, those amino acids classified as nonessential are the amino acids that are present at relatively high concentrations in the female reproductive tract (Miller and Schultz, 1987). In contrast, the amino acids

Table 7. Biphasic effect of different level of essential amino acids (EAA) and non-essential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the development of *in vitro* fertilized porcine embryos

Level of EAA and NEAA (μl/ml)		Embryo Development (%), N (Mean±SD)					
NEAA for 1st 72 h	EAA for last 4 d	N	2-4 cell	8-16 cell	Morula	Blastocysts	Hatched blastocysts
0	0	237	198 (82.3±8.9)	133 (53.4±15.3)	77 (31.9±6.7)	40 (16.9±2.1)	3 (0.9±1.9)
5	5	212	175 (81.8±8.8)	123 (56.5±10.6)	77 (35.5±5.8)	38 (18±1.6)	3 (1.2±1.4)
10	10	212	184 (85.7±9.7)	126 (59.3±15.4)	67 (32.3±12.0)	38 (18.3±3.7)	1 (0.4±0.7)
20	20	216	195 (90.1±3.7)	144 (65.4±10.2)	70 (32.4±4.5)	44 (20.3±3.9)	3 (1.4±1.9)

N = Number of oocytes. SD = Standard deviation.
Data were obtained after eight replications.
[a,b] Within the same column, values with different superscripts were significantly different ($p<0.05$).

Table 8. Biphasic effect of different levels of essential amino acids (EAA) and non-essential amino acids (NEAA) in North Carolina State University (NCSU)-23 medium on the number of inner cell masses (ICM), trophectoderms (TE) and total number of cells of porcine *in vitro* fertilized embryos

Level of EAA and NEAA (μl/ml)		Number of cells (Mean±SD)*			
NEAA for 1st 72 h	EAA for last 4 d	ICM	TE	Total	TE/ICM
0	0	16.2±3.6	32.3±6.6	46.8±8.1[b]	2.0±0.4
5	5	18.7±2.6	45.5±12.7	64.2±13.4[a]	2.5±0.8
10	10	20.5±4.9	41.3±17.4	62.0±20.6[ab]	2.0±0.7
20	20	18.7±5.4	48.5±6.1	67.2±7.6[a]	2.8±1.0

* Values shown are obtained with 15 embryos from three replicates.
[a,b] Within the same column, values with different superscripts were significantly different ($p<0.05$).

classified by Eagle (1959) as essential for somatic cells during culture are present in the reproductive tract at low concentrations (Miller and Schultz, 1987). Addition of these essential amino acids to the culture medium inhibits development of cleavage stage embryos *in vitro* (Gardner and Lane, 1993). The present study also demonstrates that supplementation of different levels of essential amino acids in the NCSU-23 medium decreased cleavage, morula and blastocyst development and ICM numbers. It has been observed that reducing the concentration of essential amino acids for development of the cow embryo *in vitro* increased blastocyst development (Steeves and Gardner, 1996). However, the viability of these embryos was not assessed. This finding is in agreement with the findings of the present study. In a study by Lane et al. (2001) stated that lowering the essential amino acid concentration decreased ammonium production in the medium in the case of mouse. Therefore, reducing the essential amino acid concentration should result in a reduction of ammonium generation in the culture medium and further enhance embryo development.

An explantation for the increased development observed when embryos were cultured with a 1/2 concentration of Eagle's essential amino acids is that reducing the concentration of amino acids in the medium decreases the amount of ammonium produced. Amino acids spontaneously break down to ammonium when incubated at 37°C (Gardner and Lane, 1993). In addition, ammonium is a byproduct of amino acid metabolism (Gardner and Lane, 1993). The levels of ammonium produced in the culture medium containing all Eagle's amino acids after incubation for only 48 h is inhibitory to embryo development (Gardner and Lane, 1993). However, essential amino acids stimulate the development of the inner cell mass (ICM) of cultured blastocysts and therefore enhance fetal development after transfer (Lane and Gardner, 1997; Gardner and Lane, 1998). This contradicts the finding of the present study.

In the case of the combined effect of essential and nonessential amino acids, better and significant results were found in blastocysts, hatching blastocysts and in ICM number which were also dose dependent. This is in agreement with the findings of Steeves and Gardner (1999) where they found that combination of all 20 amino acids stimulated blastocyst development, total cell number, the number of cells in the TE and ICM, and allocation of cells to the ICM. This is a significant finding for the optimization of culture medium, as the number of cells in the blastocyst (total) and the ICM have been positively correlated with blastocyst viability (Lane and Gardner, 1997; Papaioannou and K. M. Ebert, 1986). In fact, based on the present study, blastocysts with the highest proportion of cells in the ICM resulted from embryos being cultured in the presence of essential and nonessential amino acids.

With respect to the biphasic effect of nonessential and essential amino acids, nonessential amino acids increased the cleavage rate whereas essential amino acids increased the total cell numbers. Neither the nonessential nor the essential group of amino acids, on their own, affected blastocyst cell number or the differentiation of cells in the blastocyst. The essential amino acids did not stimulate development of the ICM, as was found in the case of mice by Lane and Gardner (1997). In the present study, the effect

of biphasic nonessential and essential amino acid supply was only significant for the total cell number.

In conclusion, this study determined the role of nonessential and essential amino acids in the culture of the porcine embryo and showed that the embryo requires different levels of amino acids as it develops from the zygote to the blastocyst. Development of the early cleavage to blastocyst stages was stimulated by the nonessential amino acids, while essential amino acids had a negative affect on embryo development. Culture with all 20 amino acids increased blastocyst formation, cell number, and the differentiation of cells into the ICM and TE. Further, the results suggest that a reduction in the concentration of essential amino acids would be beneficial to the culture of the porcine embryo. Overall, this study highlights the need to consider the species and the changing requirements of the embryo as it develops from the zygote to the blastocyst when one is optimizing a culture medium.

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