

Effects of Supplementation with Amino Acids on *in vitro* Buffalo Embryo Development in Defined Culture Media

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Abstract: The objective of this study was to investigate the effect of essential (Ess) and non-essential amino acids (NEss), on *in vitro* buffalo embryonic development in a simple defined culture medium. The selected cumulus oocyte complexes (COCs) with a compact cumulus cells and evenly granulated ooplasm were *in vitro* matured at 39°C in an atmosphere of 5% CO₂ with high humidity. After 24 h of culture, oocytes were used for *in vitro* fertilization (IVF). Following 18 h of insemination, oocytes were randomly assigned to investigate, the effect of amino acids addition to the culture media from the zygote to day 7 post-insemination (pi) (experiment 1), from day 3 of culture to day 7 pi (experiment 2), different Ess concentrations from the zygote to day 7 pi and the effect of substituting synthetic macromolecules PVP for BSA or FCS in 20aa supplemented defined medium. The current data revealed that, nonessential amino acids with glutamine (NeGln), when added to synthetic oviduct fluid media (SOF) for the first 72-h culture increased ($P < 0.05$) development to the morula stage and subsequent blastocyst development. The addition essential amino acids with glutamine (EssGln) to SOF, for the first 72-h culture, decreased ($P < 0.05$) development to the morula stage and subsequent blastocyst development. Whereas, the addition of EssGln to medium already containing NeGln (20aa) for the first 72-h culture enhanced the development to the morula stage and subsequent blastocyst development. Beyond day 3 pi, culture with EssGln increased blastocyst development nearly similar to that cultured in NeGln or 20aa. Furthermore, during the first 72-h culture, high concentration of Ess (2%) had a detrimental effect on embryo development ($P < 0.05$). In conclusion, the current results demonstrate that development of the early cleavage stages was stimulated by the nonessential amino acids and glutamine, while development beyond day 3 pi was stimulated by a combination of the nonessential and essential amino acids and glutamine. Further, the results suggest that reduction of essential amino acids concentration would be beneficial to culture of the buffalo embryo.

Key words: Amino acids % Buffalo % *In vitro* maturation % Fertilization % Embryos development

INTRODUCTION

Lack of knowledge concerning buffalo embryo physiology and reduced viability of cultured embryos limits the usefulness of assisted reproductive technologies for the genetic management of buffalo populations. During the past several years, there have been substantial efforts to optimize culture conditions for the *in vitro* production of buffalo embryos. Through the use of simple, defined culture media, it has been possible to examine the embryo's requirements for specific energy substrates such as glucose, pyruvate, lactate and glutamine [1]. There has been renewed interest on the need for and role of amino acids in chemically defined

media for the culture of preimplantation mammalian embryos [2, 3]. Essential and/or non-essential amino acids are commonly added to serum-supplemented or serum-free culture media used for mammalian embryo development *in vitro*. Given the effects of essential and non-essential amino acids on embryonic physiology, it is not surprising that blastocyst development is improved in many species by culture in relatively simple media containing optimized concentrations of amino acids [4, 5]. Apart from amino acids used for protein synthesis, they play important role as osmolytes [6], intracellular buffers [7], heavy metal chelators and energy sources as well as precursors for versatile physiological regulators, such as nitric oxide and polyamines [8]. Moreover, amino

acid profiling could help in choosing the best embryos for *in vitro* fertilization (IVF) cycles [9]. A major step toward ameliorating media for the culture of bovine embryos was the discovery that the addition of Eagle's amino acids improved embryo development [10]. Several studies had explained the way that amino acids affect mammalian embryo development and subsequent viability, on the hamster [11], mouse [12] and rat [13]. These studies have demonstrated that amino acids can be either stimulatory or inhibitory to *in vitro* embryo development. Although it has been shown that amino acids support rabbit [14], porcine [15] and bovine [16] embryo development, amino acids requirement for *in vitro* buffalo embryo development is not fully understood. The current study aimed to investigate the effects of essential and non-essential amino acids from day 0 or from day 3 of culture on the buffalo embryonic development *in vitro*.

MATERIALS AND METHODS

***In vitro* Oocytes Maturation:** Ovaries from buffalo were collected at local abattoir (El-Monie), shortly after slaughter and transported to the laboratory within 2 h in sterile normal saline containing 100 IU/ml penicillin and 100µg/ml streptomycin at approximately 35°C. Immature cumulus-oocyte complexes (COCs) were aspirated from medium-sized (2 to 8 mm) follicles with 18 G needle connected to a 10 ml sterile disposable syringe. Oocytes with more than two layers of compact cumulus cells and homogeneous granular ooplasm were selected for *in vitro* maturation, according to Totey *et al.* [17]. The COCs were rapidly washed twice in modified Dulbecco's phosphate-buffered saline, before transferring to the maturation media. The basic medium for oocyte maturation is a modified synthetic oviduct fluid media (SOF) supplemented with 10 µg/ml Luteinizing hormone, 5 µg/ml follicle stimulating hormone and 1 µg/ml estradiol-17β (Humegon; Organon, Scarborough, ON, Canada). The oocytes were cultured for 24 h at 39°C in an atmosphere of 5% CO₂ in air with maximum humidity.

Sperm Preparation and IVF: Three straws of frozen buffalo semen were thawed in a water bath at 37°C for 30 seconds. The most motile spermatozoa were separated by swim up technique in the fertilization medium, modified Tyrode's albumin lactate pyruvate medium (Sp-TALP) containing 6 mg/ml bovine serum albumin (Sigma), for 1 h according to Parrish *et al.* [18]. The uppermost layer of the medium containing the most motile spermatozoa was collected and washed twice by centrifugation. The sperm

pellet was resuspended in the fertilization TALP medium containing 10 µg/ml heparin for *in vitro* sperm capacitation. Following oocytes maturation, COCs were washed three times with the fertilization medium and then sperm/oocytes were incubated in 50 µl droplets of the fertilization TALP medium (mTALP) under sterile mineral oil at 39°C in 5% CO₂ in air with maximum humidity. The final sperm concentration of 2 x 10⁶ sperm/ml was used for oocyte insemination and ten oocytes were used for each 50 µl droplet.

Embryo Developmental Culture: At 18 h pi, presumptive zygotes were freed from loosely bound spermatozoa and remaining attached cumulus cells by gentle pipetting. Denuded zygotes were washed three times with the culture media (SOF) and then randomly allocated to treatments (experiments 1, 2, 3 and 4). All subsequent culture periods were performed in an atmosphere of 5% CO₂ in air with maximum humidity at 39°C. The culture medium was changed at 48 h intervals until day 7 pi. The proportional of cleaved oocytes was recorded 48 h pi and those developed to the morula and blastocyst stages were recorded at 5-7 day pi.

Experimental Design

Experiment 1: Influence of essential and nonessential amino acids on embryo development *in vitro* from the zygote to day 7 pi: Presumptive zygotes (18 h pi) were randomly allocated to one of five media: 1) SOF (control), 2) Gln (SOF with 1 mM glutamine), 3) EssGln (SOF with 1 mM glutamine and 1% MEM essential amino acids), 4) NeGln (SOF with 1 mM glutamine and 1% MEM nonessential amino acids), or 5) 20aa (SOF with 1 mM glutamine, 1% MEM nonessential amino acids and 1% MEM essential amino acids).

Experiment 2: Influence of essential and nonessential amino acids on *in vitro* embryo development from day 3 to day 7 pi: Presumptive zygotes (18 h pi) were cultured in NeGln for 72 h pi. Embryos were then divided equally among one of five media: 1) control, 2) Gln, 3) EssGln, 4) NeGln or 5) 20aa. Embryos were then cultured for a further 96 h to day 7 pi.

Experiment 3: Influence of different essential amino acids concentration on *in vitro* embryo development from the zygote to day 7 pi: Presumptive zygotes were randomly cultured in the SOF media with NeGln, containing (0.25, 0.50, 0.75, 1 or 2%) concentrations of MEM essential amino acids.

Experiment 4: Influence of simple defined media supplemented with 20aa from the zygote to day 7 pi on embryo development *in vitro*: Presumptive zygotes (18 h pi) were randomly cultured in the SOF+20aa and that supplemented either by, defined synthetic macromolecules 3 mg/ml polyvinylpyrrolidone (PVP) or protein, 10% fetal calf serum (FCS) or 8 mg/ml Bovine serum albumin, fraction-V (BSA-V). COCs cultured without amino acids or protein supplementation served as control.

Statistical Analysis: All data were analyzed by using Costat Computer Program (1986), Cottort Software and were compared by the least significant difference least (LSD) at 1 and 5% levels of probability. The results were expressed as means ± S.E.M.

RESULTS

Results presented in Table 1 demonstrated that, culture for the first 72 h in NeGln or 20aa increased (P<0.05) cleavage (49.53 and 51.75%, respectively), morula (28.69 and 30.46 %, respectively) and subsequent blastocyst development (22.16 and 24.16%, respectively) as compared with the control (29.60, 14.19

and 5.79%, respectively), glutamine (31.83, 14.04 and 6.32%, respectively) and EssGln (33.58, 15.61 and 9.29%, respectively) supplemented groups. During the first 72 h of culture, 1% essential amino acids had a detrimental effect on embryo development *in vitro*.

Results presented in Table 2 showed that, culture from day 3 to day 7 pi, in SOF supplemented with NeGln, EssGln or, 20aa increased (P<0.05) cleavage, morula and subsequent blastocyst development as compared with the control. Data suggested that after day 3 pi, the buffalo embryos showed a requirement for the nonessential amino acids, the essential amino acids and glutamine. The combination of all 20 amino acids stimulated blastocyst development.

Results in Table 3 revealed that, decreasing the essential amino acids concentrations during the first 72 h of culture augmented the *in vitro* embryo development. Culture of presumptive zygotes in SOF+NeGln with 0.5% essential amino acids increased (P<0.05) cleavage, morula and subsequent blastocyst development (63.89, 42.33 and 28.53%, respectively) compared with the control and other essential amino acids concentrations. During the first 72 h of culture, high concentrations of Ess had a drastic effect on *in vitro* embryo development, even in the presence of nonessential amino acids and glutamine.

Table 1: Influence of essential and nonessential amino acids on embryo development *in vitro* from the zygote to day 7 pi

Treatments	No. Presumptive zygotes	Cleavage rate	Morula stage	Blastocyst stage
Control	72	29.60±4.32 ^b	14.19±2.43 ^b	5.79±1.87 ^b
SOFGln*	62	31.83±2.85 ^b	14.04±3.46 ^b	6.32±1.06 ^b
EssGln**	75	33.58±3.88 ^b	15.61±3.27 ^b	9.29±2.14 ^b
NeGln***	73	49.53±1.67 ^a	28.69±3.49 ^a	22.16±4.14 ^a
20aa****	78	51.75±5.43 ^a	30.46±1.76 ^a	24.16±1.26 ^a

* Gln: Glutamine **Ess= Essential amino acids *** NEss= Non-essential amino acids ****20aa=All amino acids

^{ab} Different superscripts within column indicate significant differences (at least P < 0.05).

Table 2: Influence of essential and nonessential amino acids on *in vitro* embryo development from day 3 to day 7 pi

Tre Treatments	No. Presumptive zygotes	Cleavage rate	Morula stage	Blastocyst stage
Control	72	26.34±2.78 ^b	15.10±1.79 ^c	7.03±1.55 ^b
SOFGln*	68	34.07±4.55 ^a	17.76±2.75 ^c	10.29±1.42 ^b
EssGln**	74	45.78±2.65 ^a	25.43±3.52 ^b	21.20±1.69 ^a
NeGln***	77	47.89±3.17 ^a	29.77±1.23 ^b	23.59±1.49 ^a
20aa****	69	53.57±2.48 ^a	37.69±2.42 ^a	27.58±3.65 ^a

*Gln: Glutamine **Ess= Essential amino acids *** NEss= Non-essential amino acids ****20aa=All amino acids

^{ab} Different superscripts within column indicate significant differences (at least P < 0.05).

Table 3: Influence of different essential amino acids concentration on *in vitro* embryo development from the zygote to day 7 pi

Tre Treatments	No. Presumptive zygotes	Cleavage rate	Morula stage	Blastocyst stage
Control	82	29.11±2.73 ^{cd}	14.81±2.68 ^c	8.59±1.32 ^{cd}
Ess 0.25%	92	48.09±2.75 ^b	28.93±3.54 ^b	19.16±3.13 ^b
Ess 0.50%	81	63.89±3.55 ^a	42.33±3.27 ^a	28.53±1.73 ^a
Ess Ess 0.75%	93	38.91±1.98 ^{bc}	18.58±2.78 ^c	12.54±1.63 ^c
Ess 1%	93	27.60±6.43 ^{cd}	16.26±2.73 ^c	8.53±2.09 ^{cd}
Ess 2%	87	18.18±2.05 ^d	12.48±1.58 ^c	5.69±1.02 ^d

^c Ess= Essential amino acids

^{c,ab} Different superscripts within column indicate significant differences (at least P < 0.05).

Table 4: Influence of simple defined media supplemented with 20aa from the zygote to day 7pi on embryo development *in vitro*

Tre Treatments	No. Presumptive zygotes	Cleavage rate	Morula stage	B Blastocyst stage
Control	82	30.21±3.18 ^b	13.18±2.73 ^b	5.97±2.19 ^b
FCS*	72	48.72±5.31 ^a	26.59±4.35 ^a	18.25±4.15 ^a
BSA**	83	33.56±3.61 ^b	21.60±1.42 ^{ab}	10.73±1.76 ^{ab}
PVP***	85	46.96±1.73 ^a	26.29±3.96 ^a	16.79±4.53 ^{ab}

*FCS= Fetal calf serum **BSA= Bovine serum albumin ***PVP= polyvinylpyrrolidone

^{c ab} Different superscripts within column indicate significant differences (at least P < 0.05)

Results presented in Table 4 pointed out that SOF+20aa supplemented with defined macromolecules (PVP) supported embryo development *in vitro* in a rate similar to that supplemented with BSA or FCS, as indicated by the morula and blastocyst yield.

DISCUSSION

Culture media components and culture conditions can affect and even modulate the *in vitro* development of mammalian embryo [19]. It is therefore necessary to devise and optimize culture systems that take into account all the factors essential for *in vitro* embryo development. *In vivo*, the mammalian embryo is exposed to significant levels of amino acids (Ess and NEss) in oviduct and uterine fluids [20]. Specific amino acid transporters are present on the membranes of oocytes and embryos and a supply of amino acids for protein synthesis is essential for normal embryo growth [21]. The present study revealed that the buffalo embryo has a requirement for amino acids during development from the zygote to the blastocyst. The current results were in accordance with results of previous studies that have established the beneficial effect of amino acids addition to the culture media compared with defined media without amino acids supplementation [22]. Furthermore, Gardner *et al.* [23] reported that, amino acids addition to the culture media reduced the percentage of embryos arrested during culture and stimulated both cleavage and hatching by the increase of endogenous amino acid pool sizes and/or de novo protein synthesis.

The current study showed that culture for the first 72 h in NeGln stimulated cleavage to the morula stage and subsequent blastocyst development. During this period, EssGln and glutamine as the sole amino acid had a drastic effect on embryo development. The fact that NeGln increased cleavage is important for the optimization of culture media, as an increased rate of development of the early cleavage stage embryo has been correlated with an increase in viability [12, 24]. The current results were in accordance with results of previous studies that have demonstrated the stimulatory effects of the

combination of nonessential amino acids and glutamine in development of mouse [25] and ovine [23] embryos *in vitro*. This improvement may be attributed to, the NeGln stimulate embryonic development by decreasing the time of the first three cleavage divisions [26]. However the current results were disagreed with the findings of Rosenkrans *et al.* [27] and Rezaei and Chian [22] who found that Ess alone tended to increase blastocyst development. Furthermore, Pinyopummintr and Bavister [28] found that development of the bovine embryo to the 8- to 16-cell stage was equivalent after culture in the presence of nonessential amino acids (media also contained glutamine), essential amino acids, or glutamine alone.

The current data verified that, development to the morula stage and subsequent blastocyst development was equivalent for embryos that had been cultured for the first 72 h in 20aa or NeGln. These results were in accordance with previous studies in cattle [29] and sheep [23] that have shown that the presence of the essential amino acids did not negate the stimulatory effects of the nonessential amino acids and glutamine. This may indicate that the transport mechanisms for nonessential and essential amino acids differ in cattle, sheep and buffalo. While, the current results were in contrast with the findings of Lane and Gardner [12] who found that, culture of the mouse embryo up to the 8-cell stage with essential amino acids negated the stimulatory effect of the nonessential amino acids and glutamine, reducing subsequent blastocyst cell number and pi development. This discrepancy might be due to a species-specific or stage-specific requirement for amino acids during oocyte maturation and embryo development.

The present data showed that after day 3 pi, the buffalo embryos showed a requirement for the nonessential amino acids, the essential amino acids and glutamine. The combination of all 20aa augmented the *in vitro* embryo development, these results were in consistent with the previous studies [30, 31] showing that the 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 enhanced developmental potential may be caused by the alleviation of osmotic stress on the ova and zygotes by the amino acids that are osmolytes. Lane and Gardner [32] found that each group of amino acids had quite specific functions in the development of the mouse blastocyst. The nonessential amino acids and glutamine stimulated blastocyst formation and hatching, while the essential amino acids stimulated cleavage, differentiation of cells to the ICM and fetal development after transfer. These results may be explained by the findings of Steeves and Gardner [24] and Lane and Gardner [32] who revealed not only that the bovine embryos has a requirement for amino acids, but also that amino acids have both a temporal and differential effect during development from the 2-cell zygote to the blastocyst stage. They suggest that the requirement for amino acids changes according to the developmental stage of embryos and the metabolic requirements are different during different developmental stages.

The current results demonstrated that, a reduction in the concentration of Eagle's essential amino acids (when in combination with the nonessential amino acids and glutamine) improved the *in vitro* embryo development to the blastocyst stage. The optimal concentration of essential amino acid in the present study appeared to be 0.50 %, whereas high essential amino acid concentration 2% resulted in drastic decrease in embryo development. These results were in consistent with Suzuki and Yoshioka [33] who found a significant inverse correlation between essential amino acids concentrations and embryo cleavage and subsequent blastocyst development. Moreover, the amino acids present in Eagle's MEM essential amino acids are at higher concentrations than are found in the ruminant reproductive tract [34]. Bavister and McKiernan [35] found that a reduction in the concentration of essential amino acids for culture of the hamster embryo changed them from inhibitory to stimulatory amino acids. Liu and Foote [29] reported that culture of bovine embryos from the 4-cell stage to the blastocyst with half the concentration of essential amino acids resulted in a significant increase in the proportion of hatching blastocysts. The detrimental effect of high concentration of essential amino acids on embryo development may be attributed to, amino acids have been shown to spontaneously break down in culture to produce ammonium [36, 37]. Also, the embryo metabolizes amino acids, resulting in the additional production of ammonium in the medium [36]. Ammonium in the culture medium has

been shown to be detrimental to blastocyst development, significantly reduces blastocyst cell number, decreases inner cell mass development, increases apoptosis, perturbs metabolism and impairs the ability of embryos to regulate intracellular pH [38].

The present study showed that, synthetic defined macromolecules 3 mg/ml PVP with 20aa supplemented medium enhanced the developmental potentials of buffalo embryos to the blastocyst stage comparable to that cultured in a media supplemented with BSA or FCS. These results were in agreement with Boni *et al.* [39], who indicated that defined media could support the embryo development.

In conclusion, this study emphasizes that development of the early cleavage stages was stimulated by the nonessential amino acids and glutamine, while development beyond day 3 pi was stimulated by a combination of the nonessential and essential amino acids and glutamine. Culture with all 20 amino acids increased embryo development to the blastocyst stage. Further, the results revealed that a reduction in the concentration of essential amino acids would be beneficial to culture of the buffalo embryo. Buffalo presumptive embryo can be successfully developed in a chemically defined medium supplemented with 20aa and PVP.

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