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Supplementation of Amino Acids in Serum Free IVM-Media and its Effect on *In Vitro* Maturation and Fertilization of Buffalo Oocytes

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AMINO acids are an important component of culture media and their beneficial role is reported in different species. It was hypothesized that supplementation of amino acids to *in vitro* maturation (IVM) media may affect IVM and fertilization of buffalo oocytes. The objective was to study the effect of supplementing IVM media with essential amino acids (EAA), non-essential amino acids (NEAA) or both (EAA+NEAA) on *in vitro* maturation (Experiment I) and fertilization (Experiment II) of buffalo oocytes. In experiment I, cumulus-oocyte complexes aspirated from follicles (2-8 mm in diameter) were matured in IVM media: (1) Maturation medium (MM; TCM-199 enriched with BSA 6mg/mL, 1 µg/mL estradiol-17β, 10 IU/mL LH, 0.5 µg/mL FSH and 50 ug/mL gentamicin) alone as control; (2) MM supplemented with 2% EAA solution; (3) MM enriched with 1% NEAA solution; and (4) MM added with 2% EAA solution + 1% NEAA solution at 38.5°C in an atmosphere with 5% CO_2 and 95% humidity under mineral oil.. The degree of cumulus expansion and degree of nuclear maturation (Aceto orcein staining) was measured after 24 h of incubation. In experiment II, following maturation as in experiment I, the oocytes were co incubated with sperms at 39 °C in 5% CO_2 in air with maximum humidity. After 18 h, oocytes were stained as described earlier and sperm penetration rate was recorded. Cumulus expansion was higher ($P<0.05$) in medium supplemented with EAA, NEAA and EAA+NEAA compared to control. Nuclear maturation rate and Sperm penetration rate remained similar in media supplemented with EAA, NEAA, EAA+NEAA and control. Results indicated that supplementation of maturation media with amino acids have beneficial effect on oocyte maturation in terms of cumulus expansion. However, non-significant effect on sperm penetration rate suggested further studies to evaluate the effect of amino acids presence of *in vitro* maturation media on subsequent culturing of embryos.

Keywords: Amino acids, Buffalo, Oocytes, *in vitro* maturation, Serum free media.

Introduction

Buffalos are important part of livestock industry in Pakistan and are considered as black gold of Pakistan [1]. However, different factors such as low number of follicles, poor ovulatory response and atretic follicles leads to low reproductive potential in buffalos. To deal with these problems different types of assisted reproductive techniques such as superovulation, artificial insemination and

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in vitro embryo production (IVEP) are applied. IVEP which involves the utilization of both male and female superior germplasm simultaneously is gaining more importance as compared to various reproductive techniques such as artificial insemination and superovulation [2]. IVEP provides sufficient number of embryos that can be used as experimental material for research in the field of cellular and molecular embryology of farm animals and to study the factors effecting the early embryonic development [3].

In vitro embryo production (IVEP) involves three steps, *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culturing (IVC) [4-6]. *In vitro* oocytes maturation is the crucial and challenging step towards successful *in vitro* production of buffalo embryos. The composition of maturation media affects the development of oocytes, its ability to fertilize and undergo embryonic development up to blastocyst stage [7, 8].The maturation media used for *in vitro* maturation of oocytes contains vitamins, amino acids, purines and other components that are needed for survival of cell [6]. Serum composed of various types of substances like growth factors, proteins, vitamins and many other unidentified substances is usually added to maturation medium. Some of these unidentified substances may have adverse effect on *in vitro* oocytes maturation [9, 10].

To understand the requirement of maturation media all the products with unidentified substances, such as serum should be replaced with some identified components. Complete removal of serum from culture protocol is challenging task. Serum has been transitionally replaced in mammal culture system by bovine serum albumin (BSA), polyvinyl alcohol (PVA) [11] and recently by polyvinyl pyrrolidone (PVP) [12], antioxidants, growth factors [13] and amino acids [14]. Amino acids have been successfully used in a chemically defined *in vitro* maturation media of porcine [15,16] and cattle oocytes [14].

In buffalo, amino acid supplementation has been found beneficial for culture of embryo in chemically defined medium. In this study, our hypothesis that supplementation of amino acids to serum free IVM media improves the rate of maturation in buffalo oocytes. Hence, the objective of present study was to determine the effect of essential amino acids (EAA) and non-essential amino acids (NEAA) in a serum free IVM media on maturation and subsequent fertilization of buffalo oocytes.

Materials and Methods

Slaughtered buffalo ovaries were immediately collected at Sihala Slaughter House, Islamabad. Ovaries were kept at 32 °C in a thermos with sterilized phosphate buffered saline (PBS) and transferred to the laboratory within two hours of slaughter. Then ovaries were washed three times with phosphate buffered saline [17]. Cumulus oocyte complexes (COCs) were recovered from 2-8 mm follicles with 10 mL syringe attached to 18-gauge needle. Follicular fluid was pooled into a 50 mL conical tube. After collection the follicular fluid was allowed to settle for 10-15 minutes and the sediment was placed in 90 mm Petri dish. COCs were searched under stereomicroscope. Oocytes with multi layers of tightly condensed cumulus cells, unfragmented ooplasm and with intact zona were selected for further processing [18].

In vitro oocyte maturation

A chemically defined serum free medium (Maturation Media (MM): Tissue Culture Medium-199 (TCM-199; Sigma M4530) supplemented with 10 µg/mL Luteinizing Hormone (LH; Sigma L6420), 1 µg/mL estradiol-17β (Sigma E2758), 10 µg/mL Follicle Stimulating Hormone (FSH ; Sigma F8174), and 50 µg/mL gentamycin (Sigma G1272)) was used as basic maturation medium. COCs were washed twice with oocyte wash media TL Hepes (TCM-199=5ml, Bovine serum albumin (BSA) (4%) = 4mg/ml, Hepes 0.27ml of 1M Hepes (Sigma M7528), once with *in vitro* maturation (IVM) media and were assigned to four experimental groups respectively: 1) Basic maturation media supplemented with BSA. 2) Maturation media supplemented with essential amino acids (EAA Sigma B6766; 2%). 3) Maturation media supplemented with non-essential amino acids (NEAA Sigma M7145; 1%). 4) Maturation media supplemented with essential amino acids (EAA; 2%) + non-essential amino acids (NEAA; 1%). The media were sterilized with Millipore membrane filter orange scientific filter (0.22 µm Orange scientific filter). A 100µl drop for each experimental group was placed in Petri dishes (25 mm), covered with mineral oil and kept in CO_2 incubated for 2 hours at 5% CO_2 and 95% humidity prior to use. After 2 hours of incubation, 5-10 oocytes were transferred to each of four experimental groups in Petri dishes and placed in CO_2 incubator for 22-24hrs. Degree of cumulus expansion was recorded under a stereomicroscope after 24 hrs of maturation as described earlier [19].

Assessment of nuclear maturation

To assess the stage of nuclear maturation at the end of the maturation time, vortexing is done in TCM-199 containing 300IU/mL hyaluronidase for 2 min to denude the oocytes completely. Denuded oocytes were then washed twice and positioned on a grease-free slide and overlaid with a cover slip supported by two lines of a Vaseline/Paraffin mixture (40:1). Oocytes were slightly compressed onto the slide and acetic acid: ethanol fixative (1:3 v/v) was passed twice. Oocytes were then stained with Acetoorcein (1% orcein in 45% acetic acid). The stain was passed once by introduction through one side and was removed with filter paper from the other side. After 3-5 minutes destaining solution (acetic acid: distilled water: glycerol 1:3:1) was passed in a similar way to remove excess stain. Oocytes were then examined under phase contrast microscope at 400X and were classified either at germinal vesicle stage (GV: oocytes arrested at prophase I with a prominent nucleolus); germinal vesicle breakdown (GVBD: chromosomes condensed with the resolution of nuclear membrane); Metaphase I (M-I: Chromosomes arranged at equator without polar body) or Metaphase II (M-II: extrusion of 1st polar body) stage of nuclear maturation.

Sperm preparation and IVF

Swim-up method was performed with Sp-TALP (Sperm tyrodes albumin-lactate-pyruvate) medium [20, 21]. Briefly, 3mL of TALP medium (pH 7.3-7.4) was poured into four 15mL tubes and incubated in an atmosphere of 5% CO_2 in air at 39°C for 2 hours prior to use. Two straws of cryopreserved buffalo semen were thawed in a water bath at 37°C for 30 seconds. Thawed semen was transferred into a 15mL tube. About 70µl of semen was transferred into each of the four tubes containing sperm TALP. The tubes were placed at 45° angle in incubator for 30 minutes. The supernatant from the four tubes was collected with Pasteur pipette, transferred into another 15mL falcon tube and was centrifuged at 1600rpm for 10 minutes. After centrifugation the supernatant was removed and sperm pellet was suspended into 0.9mL of fertilization TALP containing 10µl/mL heparin for sperm capacitation.

Sperm concentration was measured with Neubauer haemocytometer [22]. For *in vitro* fertilization, Fert- TALP was used. The pH of the media was adjusted at 7.8 and filtered through 0.22 µm Millipore membrane filter. Drops of 50µl of fertilization TALP were prepared in Petri dishes covered with mineral oil and incubated at 39 °C in 5% CO_2 for two hours prior to use. Following oocytes maturation, expanded COCs were washed three times with the fertilization TALP and transferred to 50µl droplets. 5-10 oocytes were placed in each droplet and sperms were co incubated with 1 x 106 sperm cells in each droplet. The fertilization droplets were incubated for 18 hours at 39 °C in 5% CO_2 in air with maximum humidity.

After the completion of 18 hours of insemination, the fertilized oocytes were stained by procedure as described above. Oocytes are considered to be normally fertilized if oocytes were obtained with one penetrating sperm or having both male pronucleus (MPN) and female pronucleus (FPN). If oocyte is found with more than two pronuclei then condition is said to be polyspermic which is not considered as normal.

Statistical analysis

To find out the difference between the treatments during maturation on cumulus expansion, nuclear maturation and in *vitro* fertilization, analysis of results is done by chi square test. A 5% significance level was used. Data were analyzed with Minitab v. 15.

Results

In vitro maturation of oocytes

Cumulus expansion

Degree of cumulus expansion was categorized as not expanded cumulus, partially expanded and fully expanded (Fig. 1). The percentage of oocytes with fully expanded cumulus in media supplemented with 2 % EAA, 1 % NEAA and both 2 % EAA, 1 % NEAA did not differ ($P>0.05$) but remained higher than percentage of oocytes with fully expanded cumulus in control ($P<0.05$). (Table 1). In present study, most of the COCs in maturation media supplemented with 2% EAA or/ and 1% NEAA showed fully expanded cumulus.

Nuclear Maturation of Oocytes

Stages of nuclear maturation were classified as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) (Fig. 2). The stages of nuclear maturation did not differ significantly ($P>0.05$) between IVM media supplemented with EAA, NEAA, EAA + NEAA and control (Table 2).

In vitro fertilization of oocytes

Stages of *in vitro* fertilization were classified as oocytes 1) penetrated having swollen sperm

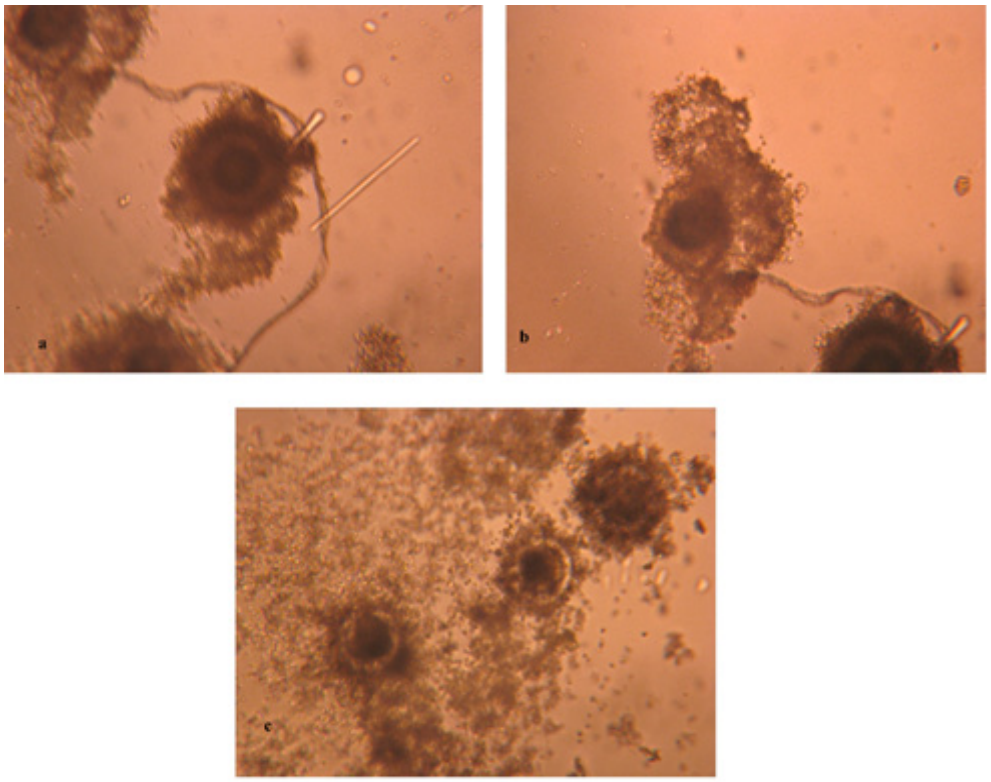

Fig. 1. Degree of cumulus expansion after 24 hrs of IVM (a). Not expanded cumulus layer (b). Partially expanded cumulus layer (c). Fully expanded cumulus layer

TABLE 1. Effect of four different treatments on degree of cumulus expansion of buffalo oocytes after 24hrs of *in vitro* maturation

IVM Media	No. of oocytes	Degree of cumulus expansion n (%)		
		Not expanded	Partially expanded	Fully expanded
Maturation media (MM)+BSA	371	67(18.05)[a]	130(35.04)	174(46.90)[a]
MM+ 2 %EAA	362	42(11.60)[b]	104(28.72)	216(59.66)[b]
MM+ 1%NEAA	370	32(8.64)[b]	111(30)	227(61.35)[b]
MM+ 2%EAA+ 1%NEAA	361	30(8.31)[b]	106(29.30)	225(62.25)[b]

*Values within a column with different superscript differ (P<0.05)
MM: Maturation media BSA: Bovine serum albumin
EAA: Essential amino acids NEAA: Non-essential amino acids

head and/or with female pronucleus (FPN), with 2PN (male pronucleus; MPN and FPN) or with three pronucleus (two sperms) or more considered polyspermic (Fig. 3). Table 3 shows the effect of IVM-medium supplement with 2% essential and 1% nonessential amino acids on subsequent in-vitro fertilization. There were no significant differences in penetration rates among the four groups. In present study proportion of oocytes with MPN was non- significantly (P>0.05) higher in oocytes matured in 1% NEAA from oocytes matured in media supplemented with EAA, EAA + NEAA and control. Also, percentage of oocytes with polyspermy was low in oocytes matured in medium supplemented with both 2%

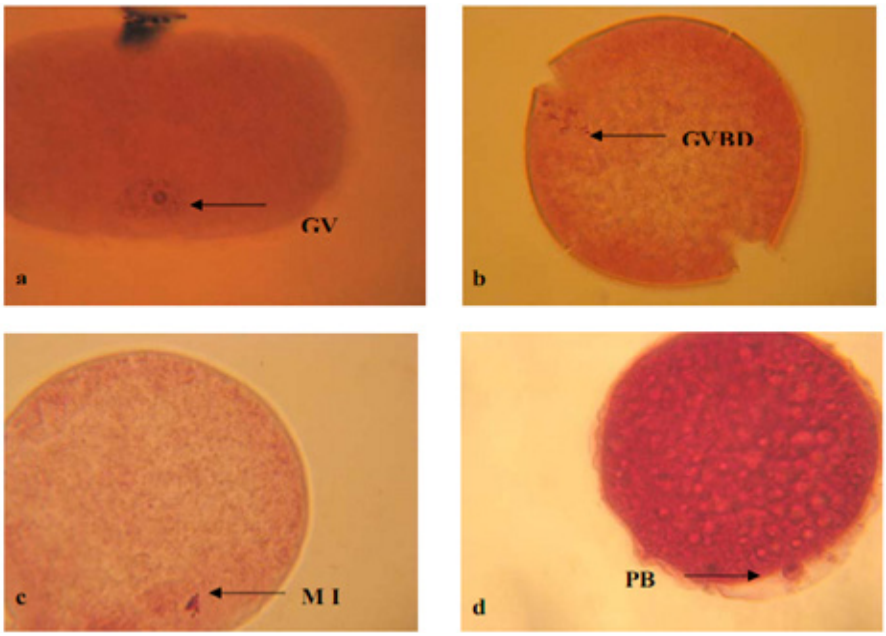

Fig. 2. Stages of oocytes nuclear maturation after 24hrs of IVM(a). Germinal vesicle stage (GV), clearly showing nucleolus (b). Germinal vesicle breakdown stage, GVBD (arrow) showing condensation of chromosomes c. Metaphase I, condensed chromosome at meiotic plate without polar body d. Metaphase II stage, extrusion of first polar body (PB)

TABLE 2. Effect of four different treatments on nuclear maturation of buffalo oocytes after 24hrs of *in vitro* maturation

Treatments	No. of oocytes	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)
MM+BSA	177	4(2.25)	46(25.98)	75(42.37)	52(29.37)
MM+ 2%EAA	183	4(2.18)	46(25.13)	82(44.80)	51(27.86)
MM+1%NEAA	189	9(4.76)	36(19.04)	82(43.38)	62(32.80)
MM+2%EAA+1%NEAA	195	1(0.51)	54(27.69)	80(41.02)	60(30.76)

Chi-square analysis, P> 0.05
MM: Maturation media BSA: Bovine serum albumin
EAA: Essential amino acids NEAA: Non- essential amino acids

EAA and 1% NEAA as compared IVM medium supplemented with 1% NEAA, 2%EAA and without amino acids(control) but the differences were not significant (P>0.05).

Discussion

The present data showed that although oocytes with fully expanded cumulus were less in control than in maturation media supplemented with essential amino acids or/and non-essential amino acids, proportion of oocytes reaching MII stage did not differ significantly among four treatments. Therefore, it is relevant to mention that there was no association between cumulus expansion and resumption of meiosis in buffalo oocytes in present study. Once previous study also reported that cumulus expansion and nuclear maturation are independent of each other and found oocytes with little cumulus expansion at MII stage [23]. However, in another previous study, some relationship between degree of cumulus expansion and oocyte development was reported in bitch [24].

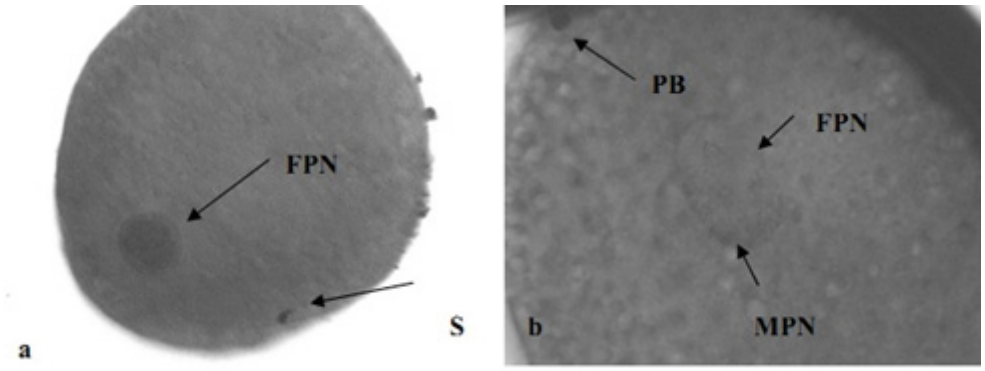

Fig. 3. Stages of *in vitro* fertilization of buffalo oocytes after 18hrs of insemination

a. Fertilized oocyte with female pronucleus (FPN) and condensed head (S) b. Normal pronuclear formation, Female (FPN) and male pronucleus (MPN) decondensed.

TABLE 3. Effect of four different treatments on *in vitro* fertilization of buffalo oocytes after 18hrs of insemination

Treatments	Number of Oocytes (%)				
	Inseminated	Fertilized	Penetrated	With 2PN	Polyspermic
MM+BSA	103	55(53.39)	17(30.90)	34(61.81)	4(7.27)
MM+2%EAA	101	64(63.36)	20(31.25)	40(62.5)	4(6.25)
MM+1%NEAA	107	72(67.28)	12(16.66)	56(77.77)	4(5.55)
MM+2%EAA+1%NEAA	103	66(64.07)	22(33.33)	42(63.63)	2(3.03)

*Chi-square analysis, P> 0.05

MM: Maturation media BSA: Bovine serum albumin

EAA: Essential amino acids NEAA: Non essential amino acids

PN: Pronucleus

Previously, it is reported that supplementation of carbohydrates with amino acids improved the maturation rate of bovine oocytes, while amino acids alone had no beneficial effect on oocytes maturation [25]. Our results are in line with results of Rezaei and Chain [14], who reported non-significant effect of EAA and/or NEAA on maturation rates of bovine oocytes. In contrary, Hong et al. [15] reported that supplementation of amino acids especially NEAA in defined mNCSU-23 medium promoted maturation and subsequent development of pig oocytes. This difference could be due to difference in species, oocyte quality, culture condition and composition of IVM media or other environmental factors.

The current results demonstrated that the effect of IVM-medium supplement with 2% essential and/or 1% nonessential amino acids during oocyte maturation has no significant differences in penetration rates among the four groups. Different studies have revealed that supplementation of amino acids in IVM medium promotes MPN formation in bovine [26] and pig [27], another study have suggested that supplementation of amino acids; especially EAA in chemically defined maturation media promotes bovine oocytes cytoplasmic maturation and subsequent embryonic development [14].

It has been investigated that delay in exocytosis of cortical granule can increase polyspermy. In current study percentage of oocytes with polyspermy was low. Hence we can suggest that buffalo oocytes matured in defined IVM media have the ability to release cortical granules. Previously study on pig oocytes reported that amino acids in defined medium supports monospermic fertilization, MPN formation and preimplantion development [15].

In conclusion, buffalo oocytes could be matured in serum free IVM medium supplemented with EAA and NEAA, without any deleterious effect on oocyte. The results of present study indicated that EAA and NEAA have beneficial effect of cumulus expansion of buffalo oocytes but had no advantageous effect on nuclear maturation and fertilization.

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