

Effect of Bovine Serum Albumin on *In vitro* Maturation, *In vitro* Fertilization and Subsequent Development of Goat Embryos

L. Asad^{1*}, M.A.M.Y. Khandoker² and A.N.M.I. Rahman¹

¹Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh.

²Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh-2200, Bangladesh.

*Corresponding author email id: lamyea_sau@yahoo.com

Abstract – The present study was undertaken to find out the effect of bovine serum albumin (BSA) on *In vitro* maturation, fertilization and to support the subsequent embryonic development of goat oocytes. Cumulus oocytes complexes (COCs) were collected from slaughter house goat ovaries by aspiration method. COCs were matured for 24 hours in TCM-199 basic medium and supplemented with different levels of BSA (2mg/ml, 4mg/ml and 6mg/ml) where 0mg/ml was considered as control. Three levels of cumulus cell expansion after 24 h of *In vitro* culture (at 38.5°C and 5% CO₂ in an incubator) observed under 10x magnification of microscope and the different stages of nuclear maturation was observed based on chromosomal configuration. Metaphase-II stages were 40.78 ± 3.84 , 67.52 ± 0.85 , 68.95 ± 1.88 and $57.74 \pm 2.39\%$. The fertility level was indicated by pronuclei formation. Normal fertilization (formation of 2 pronuclei) were 23.28 ± 3.00 , 35.52 ± 1.21 , 37.74 ± 1.24 and $29.30 \pm 3.73\%$ for 0 mg (control), 2 mg, 4 mg and 6 mg level of BSA respectively. The results indicated that the maturation and fertilization rate could be significantly increased ($p < 0.01$) by supplementing 2 mg per ml level of BSA. The rates could not be improved significantly ($p > 0.01$) by increasing the level to 4 mg per ml and when the BSA level was increased to 6 mg per ml, the rate of maturation and fertilization decreased significantly ($p < 0.01$). The development of blastocyst was found as 7.36, 12.21, 14.45 and 9.65%, respectively. The results indicate that the maturation, fertilization and subsequent embryo development rate could be significantly ($p < 0.01$) increased by supplementing 2 mg/ml of BSA and the rates could be improved further non-significantly by increasing the level to 4 mg/ml but significant ($p < 0.05$) deterioration occurred when BSA was increased up to 6 mg/ml. Thus, it can be concluded that supplementation of BSA with TCM-199 is required for regular *In vitro* fertilization and the 2 mg per ml BSA might be used as a supplementation for *In vitro* maturation, fertilization and subsequent development of goat oocytes.

Keywords – BSA, Goat Oocytes, *In vitro* Maturation, *In vitro* Fertilization and *In vitro* Culture.

I. INTRODUCTION

In vitro production (IVP) of goat embryos is a rapidly advancing field and it has been improved greatly during the past two decades [1], [2]. The production of valuable transgenic goats, capable of producing substances of pharmaceutical and technological value in their milk, meat has encouraged the development of *In vitro* techniques able to support the propagation of this animal. Before this latest development in gametes and embryo cellular biology, the field of molecular embryology of farm animals has been poorly explored due to the limited

availability of suitable experimental material at an acceptable cost. For this reasons the *In vitro* techniques for the production (IVP) of mammalian embryos have received great attention and support in recent years [3], [4].

Basically, IVP includes three major steps: *In vitro* maturation (IVM), *In vitro* fertilization (IVF) and *In vitro* culture (IVC) of the resulting embryos. However, primary oocytes collection should be added upstream of these major steps and embryo management (freezing, transfer) should be added downstream to give a complete overview of the whole process.

For successful IVP, *In vitro* maturation (IVM) is an essential step. It is well established that *In vitro* maturation of oocyte is divided into nuclear and cytoplasmic processes. Nuclear maturation involves resumption of meiosis and progression to metaphase-II stage. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed for the oocytes to be fertilized and developed into a normal embryo and offspring [5]. The cumulus cells (COCs) surrounding the oocyte plays a key role of cytoplasmic maturation. It is known to supply nutrients, energy substrates and messenger molecules for the development of oocyte [6]. Cumulus cell concentration is directly dependent on the efficiency of oocyte harvesting [7].

In vitro fertilization (IVF) is a process by which oocytes are fertilized by sperm outside the womb, *In vitro*. The process involves removing ova from the female ovaries and letting sperm fertilize them in a suitable culture medium. The fertilized oocytes are then transferred to the recipient uterus with the intent to establish a successful pregnancy. Efficiency of IVP depends on several factors, such as transport time and temperature from the abattoir to the laboratory, follicle size, developmental stage of oocyte, oocyte diameter, composition of media [8], hormones [9], serum and protein supplementation to the basic culture medium [10].

Generally buffered Tissue Culture Medium-199 (TCM-199) is used as a basic medium for IVM of goat oocytes [11]. To establish a well-defined medium, scientists added different supplements to this basic medium from different sources maintaining different level. For example, some added glutathione and glucose [12]; hormones like, gonadotrophins (FSH and LH) and estradiol-17 β [13], human chorionic gonadotrophin (hCG) in combination with FSH and estradiol-17 β . Some added growth promoting factors like vitamins or other substances [14], insulin-like growth factor-I, cysteamine [15]. Some added

protein sources like fetal bovine serum (FBS), fetal calf serum (FCS), estrus goat serum (EGS), estrus sheep serum (ESS) [16], follicular cells and bovine serum albumin (BSA).

BSA improves maturation, fertilization, blastocyst formation and hatching rates *In vitro* [17] and it has been widely used in medium for the capacitation of sperm and the acrosome reaction. It is generally believed that the beneficial effects of serum are due to cyclic adenosine monophosphate, catecholamines, vitamins, putative growth factors, lipids and albumin. It has been also demonstrated that the beneficial effect of BSA supplement is due to the presence of a relatively high molecular weight protein which contributes to maturation of oocytes. It may also have a nutritional role to play by supplementing amino acids after hydrolysis.

However, In Bangladesh, *In vitro* techniques in goat is a very recent concept [18] but a great deal of work has been done regarding evaluation and grading of ovaries, collection of COCs from slaughterhouse ovaries and grading of oocytes followed by IVM, IVF of the oocytes and IVC [19], [20]. A variety of research works have been conducted on the effects of BSA in IVM, IVF and IVC medium in bovine [21], goat [16] and hamster. There is, however, not much information regarding the efficiency of different levels of bovine serum albumin (BSA) concentration on *In vitro* maturation and fertilization of Black Bengal goat oocytes. Keeping the aforesaid reality in mind the present research was undertaken with the objective of to find out the effect of bovine serum albumin (BSA) on *In vitro* maturation, fertilization as well as subsequent development of goat embryos.

II. METHODOLOGY

A. Oocytes Collection, Harvesting and Maturation

Goat ovaries were collected from slaughterhouse. The ovaries were kept in collection vial containing 0.9% physiological saline in a thermo flask at 25°C to 30°C and transported to the laboratory within 2 to 3 hours of slaughter. The ovaries were then transferred to the sterilized petridishes containing same saline. The ovaries were rinsed thoroughly by physiological saline solution at 25°C. In the laboratory each ovary was trimmed to remove the surrounding tissues and overlying bursa. Each ovary was treated to three washings in D-PBS and two washings in oocyte harvesting medium (DPBS+4 mg/ml BSA+1.50 IU/ml Penicillin) [22]. After necessary washing, each ovary was processed individually and the oocytes harvested by aspiration technique. After aspirating the follicles from one ovary, the aspirated follicular materials were transferred slowly into a 90 mm Petri dish, avoiding damage to the cumulus cells. Then the COCs were washed 2-3 times into D-PBS before initiating the maturation experiment.

The maturation medium, tissue culture medium-199 (TCM-199) (Sigma Chemical Co., USA) was prepared and divided in to 4 groups and they were supplemented with 0mg/ml, 2mg/ml, 4mg/ml and 6mg/ml of BSA respectively, where the 0mg/ml was used as the control

group. The pH of all media was adjusted to 7.4 on the day of oocyte collection and sterilized by passage through a 20 µm sartorius Minisart filter (Toyo Roshi Co. Ltd., Japan). From each group, about 2.5-3.5 ml of the medium was poured into each of two 35 mm culture dishes. Then 1-4 drops (depending on number of oocytes) of 100 µl of medium were poured in another culture dish and covered with paraffin oil (Loba Chemie Pvt. Ltd., India). The dishes with droplet were kept in an incubator at 38.5°C with 5% CO₂ in humidified air.

After 24 hours culture of COCs in maturation medium, the level of nuclear maturation was checked. For this purpose, half of the matured COCs from each drop was taken and denuded from cumulus cells by repeated pipetting. Oocytes were then placed on a glass slide, covered with cover slip, fixed with aceto-ethanol (acetic acid: ethanol, 1:3, V/V), stained with 1% aceto-orcein. After drying, the slides were examined under inverted microscope at high magnification (100X) with emersion oil to observe the stages of maturation.

B. Semen Collection and Sperm Capacitation

Semen was collected from the bucks of Bangladesh Agricultural University artificial insemination center through artificial vagina method. Then the sperm concentration of raw semen was calculated by haemocytometer method. The semen was washed with semen washing solution though centrifuge method at 800 rpm for 5 minutes. Finally, the sperm concentration was adjusted to 2×10^6 per ml by adding semen dilution solution (BO + 2% BSA). Then 1-4 insemination droplets (100 µl) of BO medium depending on the number of the matured COCs in a 35 mm culture dish were prepared, covered with paraffin oil and were kept in the incubator for 5-6 hours for sperm capacitation.

C. Insemination and Checking the Fertilization Rate

After 24 hours of maturation, the matured COCs was proceeding to fertilization. Two 35-mm culture dishes were filled with COCs washing solution (BO + 1% BSA) and the COCs were washed 3 times. About 15-20 COCs with minimum volume of medium were transferred to each of the sperm drops prepared previously and then incubated for 5-6 hours in incubator at 38.50C with 5% of CO₂ in humidified air.

After 5-6 hours of incubation, the representative sample of the fertilized COCs from each drop was denuded from cumulus cells by repeated pipetting. Then these oocytes were fixed in a glass slide with aceto-ethanol (acetic acid: ethanol, 1:3, v/v) and stained with 1% aceto-orcein. After drying, the slides were examined at high magnification (100X with emersion oil to observe the pronucleus formation (PN).

D. *In vitro* Culture and Observation

The rest of the fertilized ova were taken from the semen drops by using glass micropipette. Then the oocytes were washed three times in pre-incubated medium (TCM-199) and were transferred to other culture drop (600µl) of TCM-199 with different levels of BSA. The dish was then kept in the CO₂ incubator at 38.5°C of 5% CO₂ in air. The development was checked every 48 hours and the culture were continued for 6 to 7 days. The number of compact m-

-orula and blastocysts were recorded on day 7.

E. Statistical Analysis

The data generated from this experiment were entered in Microsoft Excel worksheet, organized and processed for further analysis. Analysis was performed by analysis of variance (ANOVA) in completely randomized design (CRD) and for comparing means, Duncan's multiple range test (DMRT) was applied with the help of Statistical Analysis System [23].

III. RESULTS AND DISCUSSION

Experiment: 1

In experiment 1, oocytes were cultured for 24 hours in maturation medium containing various levels of BSA (0, 2, 4 and 6mg/ml). Then half of the matured oocytes from each group were checked for nuclear maturation and it was observed that the percentage of COCs reached to the Metaphase-II stages were 40.78 ± 3.84 , 67.52 ± 0.85 , 68.95 ± 1.38 and $57.74 \pm 2.39\%$. These result indicates, all BSA supplemented groups differ significantly than the non-supplemented group (Table: 1).

Experiment: 2

In this experiment, the rest half of the matured oocytes were placed in the fertilization medium (Brackett and Oliphant with different level of BSA) with the pre-incubated goat sperm. Then the rate of fertilization was checked (based on pronuclei formation) using representative sample and it was observed that the oocytes reach to normal fertilization (formation of 2 pronuclei) were 23.28 ± 3.00 , 35.52 ± 1.21 , 37.74 ± 1.24 and $29.30 \pm 0.73\%$ for 0 mg (control), 2 mg, 4 mg and 6 mg level of BSA respectively (Table:2).

Experiment: 3

After fertilization, the rest of the fertilized oocytes were transferred to the culture medium (TCM-199 with different level of BSA) for subsequent development and the observed blastocysts were 7.36 ± 1.11 , 12.21 ± 3.13 , 14.45 ± 1.74 and $9.65 \pm 1.27\%$ for 0 mg (control), 2 mg, 4 mg and 6 mg level of BSA respectively (Table: 3). In this study, we showed that it is possible to support high rates of goat embryo development *In vitro* by supplementing bovine serum albumin (BSA) with the base medium (TCM-199 for maturation, culture and Brackett & Oliphant for fertilization). In the presence of serum at all levels (2, 4 and 6 mg/ml), the maturation, fertilization and subsequent development (reach to blastocysts) were better than the non-supplemented (0mg/ml) group. BSA as a protein supplement to the original medium not only increases the rate of nuclear maturation but also enhances the development potentials of the embryos. However, extra supplementation of BSA decreases the development potential at significant rate.

Among the all supplemented groups, highest level of maturation, fertilization and blastocysts formation were observed at 4mg/ml BSA supplementation. Though, maturation, fertilization and development to blastocysts significantly increases at 2mg/ml BSA than 0mg/ml BSA but further increase in the amount of supplementation did

not enhance the rate of maturation, fertilization and subsequent development significantly, that is there was no significant difference between the 2mg/ml and 4mg/ml supplementation group. Moreover, the maturation, fertilization and blastocysts development significantly decreases when the base medium was supplemented with 6mg/ml BSA.

In recent years, research has focused on the selection of culture medium and protein supplementation for *In vitro* embryo production [24]. Mammalian embryos have been cultured in different medium including tissue culture medium with various modification. Embryos cultured in different medium and their transfer to the recipient resulted in normal offspring in domestic animal thus indicating that all chemical compounds in a medium are not necessary for *In vitro* embryo development and the selection of medium could be more empirical or even a personal preference [25]. Our result were obtained in media supplemented with different levels of BSA and there was significant difference among the different levels of supplementation, which suggested that serum supplementation with basic medium have a greater effect on *In vitro* embryo production, but after a certain stage, again supplementation reduces the embryo development significantly.

BSA has a nutritional role to play by supplementing amino acids after hydrolysis; thereby maintaining the intracellular amino acid pools. BSA also provide undefined embryo trophic (e.g. citrate, steroids) compounds, functioning as a heavy metal ion chelator/free radical scavenger, protecting cellular constituents against the effect of toxins, which enhance the fertilization of oocytes. On the other hand sperm capacitation is a prerequisite for fertilization. This capacitation is accompanied by an increase in the membrane fluidity and remodeling of the sperm surface, protein phosphorylation, and an increase in internal Ca^{2+} , pH and membrane hyperpolarization [26]. One of the processes in sperm capacitation to be recognized as fundamental in the BSA/LDH mediated cholesterol efflux—resulting in an increase of the plasma membrane fluidity, thus supporting the membrane remodeling. A positive correlation between cholesterol depletion of the sperm plasma membrane and improved capacitation of sperm has been obtained in vivo and *In vitro*. The components that act as cholesterol receptors such as albumin are present in the female tract. Thus, albumin is one of the prominent proteins supporting *In vitro* capacitation by accepting cholesterol [27]. It has been demonstrated [28] that the penetration of cumulus free oocytes in protein free media in bovine are significantly ($p < 0.05$) lower, compared with BSA contained media.

The maturation rate of goat oocytes was found comparable to the results of [29] who obtained 48-63% maturation rate in Boer goat. The maturation rate was also comparable to the results of [19] who obtained 58.57% maturation rate in Black Bengal goat. The findings of the present study were also comparable with those of sheep [22] and also suggested that the maturation rate does not depend on the collection techniques.

The nuclear maturation rate at Metaphase-II stage as 68.95% of oocytes with 4 mg/ml BSA supplementation in maturation media was comparable with the maturation rates as 63.72% obtained with 10% fetal bovine serum supplementation [16], 65.62% with 5 µg/ml FSH, 5 µg/ml LH and 1 µg/ml oestradiol, 25 mm Hepes, 0.25 mm pyruvate and antibiotics supplementation [30] and 67.24 % with 1 mg/ml estradiol and antibiotics supplementation [31].

The results of normal fertilization (2 PN formations) rate recorded in control, 2, 4 and 6 mg/ml of BSA were 23.28, 35.52, 37.74 and 29.30 %, respectively are comparable to the observation of [20] who reported 27.78-38.23% fertilization rate in goat IVF in case of 5% BSA supplementation. [32] reported that the percentage of goat oocytes with two pronuclei in IVF was 21.0-39.7% which was also in accordance with the result of present study.

The result of compact morula was comparable to the

observation of [20] who reported significantly higher ($p < 0.05$) number of compact morula and early blastocysts were obtained from grade A of 25.64% and 12.82% as compared to grade B as 6.89% and 3.44%, respectively. [33] found 31.4% and 18.6% morula and blastocyst stages of goat oocytes which were very much similar to the present result. The result observed in the present research was similar to that of [34] in some extent who obtained blastocyst yield up to 37.3% after IVF with fresh sperm capacitated without heparin and also with [35] observed blastocysts formation from 23-31%, which indicates that the culture condition is suitable for IVP of goat embryos. [36] studied sheep oocyte and found 29% rate of development to compact morula. On the other hand, 25.9% oocytes developed up to morula stage in case of goat oocytes [37] which was also similar to that of the results found in the present study.

Table 1. Effect of different level of BSA (0, 2, 4 and 6mg/ml) on *In vitro* maturation of goat oocytes

Treatment	Total number of COCs	Rate of Nuclear Maturation (%) (Mean ± SE)			
		M II	M I	GVBD	Others
0 mg/ml BSA	39	40.78 ^c ±3.84 (16)	28.11 ^a ±1.68 (11)	10.50±3.09 (4)	20.60 ^a ±2.81 (8)
2 mg/ml BSA	40	67.52 ^a ±0.85 (27)	18.46 ^b ±1.54 (7)	9.23±2.07 (4)	4.79 ^c ±2.41 (2)
4 mg/ml BSA	43	68.95 ^a ±1.88 (29)	17.68 ^b ±1.73 (8)	8.91±2.23 (4)	4.46 ^c ±2.25 (2)
6 mg/ml BSA	45	57.74 ^b ±2.39 (26)	20.13 ^b ±2.78 (9)	12.35±2.09 (6)	9.79 ^b ±1.78 (4)

Table 2. Effect of different level of BSA (0, 2, 4 and 6 mg/ml) on *In vitro* fertilization of goat oocytes

Treatment	Total number of COCs	Rate of fertilization (%) (Mean ± SE)			
		2PN	1PN	PPN	NPN
0 mg/ml BSA	38	23.28 ^c ±3.00 (9)	5.59±2.83 (2)	0.00±0.00 (2)	71.13 ^a ±1.02 (27)
2 mg/ml BSA	41	35.52 ^a ±2.21 (14)	11.29±2.53 (5)	4.76±2.38 (2)	48.89 ^b ±1.11 (20)
4 mg/ml BSA	45	37.74 ^a ±1.24 (17)	15.60±2.26 (7)	0.00±0.00 (2)	46.67 ^b ±3.33 (21)
6 mg/ml BSA	45	29.30 ^b ±3.73 (14)	5.13±2.13 (2)	2.38±1.38 (1)	63.19 ^a ±5.11 (28)

Table 3. Effect of different level of BSA (0, 2, 4 and 6 mg/ml) on *In vitro* culture of goat embryos

Treatment	Number of zygote	Development of embryos (%) at 7 days (Mean ± SE)				
		Blastocysts	Morula	8 cell	2 cell	Others
0 mg/ml BSA	30	7.36 ^c ±1.11 (2)	14.44 ^c ±1.23 (4)	20.00 ^c ±0.51 (6)	34.70 ^a ±2.75 (11)	23.05 ^a ±2.23 (7)
2 mg/ml BSA	28	12.21 ^a ±3.13 (3)	18.33 ^b ±1.67 (5)	28.91 ^a ±1.04 (8)	27.81 ^b ±1.84 (8)	13.60 ^c ±.93 (4)
4 mg/ml BSA	32	14.45 ^a ±1.74 (5)	24.89 ^a ±3.19 (8)	29.12 ^a ±1.86 (9)	24.99 ^b ±1.12 (8)	6.65 ^d ±1.41 (2)
6 mg/ml BSA	29	9.65 ^b ± 1.27 (3)	16.73 ^b ±1.42 (5)	22.68 ^b ±2.77 (7)	32.67 ^a ±1.91 (9)	18.77 ^b ±2.26 (5)

IV. CONCLUSION

The present study focused on the influence of different level of BSA supplementation on IVM, IVF and subsequent embryonic development of goat oocytes. After the above discussion, we could conclude that, considering the effects of BSA on the *In vitro* maturation, fertilization and embryo development of goat, 2mg/ml BSA can be advantageous as a supplement of maturation, fertilization and embryo culture media to increase the developmental rate of goat oocyte. Moreover, this result creates a great opportunity of conducting further research on goat embryo production.

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AUTHORS' PROFILES



Dr. Lam Yea Asad

Place and date of birth: Mymensingh, Bangladesh; 09/11/1975 Educational Background: B.Sc. in Animal Husbandry at Bangladesh Agricultural University, 1997. MS in Animal Breeding and Genetics at Bangladesh Agricultural University, Mymensingh, Bangladesh, 2001. PhD in Reproductive Biotechnology, 2016. Work Experience: Associate Professor, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Agargaon, Dhaka-1207, Bangladesh. Email: lamyea_sau@Yahoo.com



Dr. M. A. M. Yahia Khandoker

Professor, Department of Animal Breeding and Genetics Bangladesh Agricultural University, Mymensingh 2202, Bangladesh Professor (VK7) Faculty of Sustainable Agriculture, University Malaysia Sabah. Email: yahiakhabg@yahoo.com



Al-Nur Md. Iftekhar Rahman

Assistant Professor, Department of Animal Nutrition, Genetics and Breeding; Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh. B.Sc. in Animal Husbandry (Hons.); MS in Animal Breeding & Genetics; Bangladesh Agricultural University, Mymensingh 2202, Bangladesh. Email: iftekhsau@gmail.com