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## Effect of supplementation of BSA on *in vitro* maturation and fertilization of Black Bengal goat oocytes

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### ABSTRACT

The present study was conducted to find out the effect of bovine serum albumin (BSA) supplementation on *in vitro* maturation and fertilization of Black Bengal goat oocytes. Goat ovaries were collected from municipal slaughterhouse and cumulus oocyte complexes (COCs) were collected by aspiration technique. Collected COCs were matured in TCM-199 medium supplemented with different levels of BSA as 0% (control), 2.5%, 5.0% and 7.5%. Three levels of cumulus cell expansion after 27 h of *in vitro* maturation (at 38.5 °C and 5% CO<sub>2</sub> in an incubator) observed under microscope and the different stages of nuclear maturation observed based on chromosomal configuration under emersion oil at high magnification. After maturation, fresh buck semen with a concentration of 1 × 10<sup>6</sup> sperms mL<sup>-1</sup> was used for *in vitro* fertilization for 5 h at 38.5 °C and 5% CO<sub>2</sub> in an incubator. The fertility level was measured based on pronuclei formation. From this study, highest expansion was found in 7.5% BSA supplementation (75.55%), followed by 5% BSA (73.87%), 2.5% BSA (61.68%) and 0% BSA (45.36%). The percentages of COCs matured up to metaphase-II stage were 40.78, 57.74, 67.52 and 68.95 at 0% (control), 2.5%, 5% and 7.5% level of BSA, respectively. It was also observed that significantly (p<0.01) higher percentage (35.52% and 37.74%) of normal fertilization (formation of 2 pronuclei) was observed in 5.0% and 7.5% BSA level compared to control (23.28%) and 2.5% level (29.30%). These results indicated that the cumulus cell expansion, nuclear maturation and fertilization rate could be significantly increased (p<0.01) by supplementing 2.5% level of BSA. The rates could be improved further (p<0.01) by increasing the level up to 5% but no more improvement (p>0.05) occurred when BSA was increased to 7.5%. Thus, it can be concluded that bovine serum albumin at 5% level might be used as a supplement for maturation and fertilization of goat oocytes in TCM-199 medium.

**Keywords:** *In vitro* maturation, BSA, TCM-199, goat oocytes

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## 1 Introduction

*In vitro* production (IVP) of goat embryos have enormous prospect for production of huge quantity of em-

bryos and their transfer to recipients for production of normal offspring and faster development of superior germplasm. Generally, IVP comprises *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in*

*in vitro* culture (IVC) of embryos. During IVM, oocytes undergo a series of cytoplasmic changes before resumption of nuclear maturation. Nuclear maturation involves resumption of meiosis and progression to metaphase-II stage and 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. On the other hand, IVF is a process by which oocytes are fertilized by sperm outside the womb, *in vitro*. IVM and IVF of oocytes depends on several factors, such as follicle size (Blondin and Sirard, 1995), developmental stage of oocyte (Hagemann et al., 1999), serum and protein supplementation to the basic culture medium (Avery et al., 1998) and composition of media (Lonergan et al., 1997). Buffered Tissue Culture Medium-199 (TCM-199) is used as a basic medium for IVM of goat oocytes (Rahman et al., 2007). To establish a well-defined medium, scientists added different supplements to this basic medium from different sources maintaining different level. Some added protein sources like fetal bovine serum (FBS) (Tajik and Esfandabadi, 2003), estrus sheep serum (ESS) (Kharche et al., 2009), and bovine serum albumin (BSA) (Rajikin et al., 1994). Among them, it has been reported that BSA improves maturation, fertilization, blastocyst formation and hatching rates *in vitro* (Visconti et al., 1995; Bhattacharyya, 1992) and widely used in medium for the capacitation of sperm (Rajikin et al., 1994; Dow and Bavister, 1989), as well as the acrosome reaction (Yoshida et al., 1993; Andrews and Bavister, 1989). The beneficial effect of BSA supplement is due to the presence of a relatively high molecular weight protein which contributes to maturation of oocytes (Kane and Headon, 1980; Kane, 1985).

Several research works around the world have conducted research on the effects of BSA in IVM, IVF and IVC medium in bovine (Ali and Sirard, 2002; Wang et al., 1997), goat (Kharche et al., 2009; Rajikin et al., 1994) and hamster (Bavister et al., 2003). Ali and Sirard (2002) used 8 mg mL<sup>-1</sup> BSA for bovine oocytes. On the other hand, Kharche et al. (2009) performed experiment which consisted of *in vitro* matured goat oocytes co-incubated with sperm in a 50 µL drop of TALP medium containing 6 mg mL<sup>-1</sup> crystalline bovine serum albumin (BSA) fraction V and 10 µg mL<sup>-1</sup> heparin in one group, 3 mg mL<sup>-1</sup> crystalline BSA fraction V, 10% estrous goat serum and 10 µg mL<sup>-1</sup> heparin in group 2 and 6 mg mL<sup>-1</sup> fatty acid free BSA and 10 µg mL<sup>-1</sup> heparin in group 3.

In Bangladesh, IVP technology in goat is new but some research works have already been done regarding evaluation and grading of ovaries, collection of the cumulus oocyte complexes (COCs) from slaughterhouse ovaries and grading of oocytes followed by IVM, IVF of the oocytes and IVC (Mondal et al., 2008; Islam et al., 2007). However, limited information is

available regarding the efficiency of only BSA supplementation in maturation medium in Black Bengal goat oocytes. Therefore, our aim was to establish a suitable economic protocol by observing the effect of supplementation of BSA at different levels on *in vitro* maturation and subsequent fertilization of Black Bengal goat oocytes.

## 2 Materials and Methods

The experiment was conducted at the Reproductive Biotechnology Laboratory under the Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh.

### 2.1 Collection of ovaries

Goat ovaries of unknown reproductive history were collected from municipal slaughterhouse, Mymensingh in Physiological saline solution (0.9% NaCl) and transported to the laboratory at 25-30 °C within 2-3 h of collection. Each ovary was trimmed to remove the surrounding tissues and overlying bursa and treated to three washings in D-PBS and two washings in oocyte harvesting medium (DPBS + 4.0 mg mL<sup>-1</sup> BSA + 1.50 IU mL<sup>-1</sup> Penicillin) according to Wani et al. (2000).

### 2.2 Processing of COCs

The cumulus oocyte complexes (COCs) were aspirated from 2-6 mm diameter surface follicle with hypodermic needle (18G) attached to a sterile 5 mL disposable plastic syringe. The aspirated follicular materials were transferred slowly into a 90 mm Petridish and the COCs were searched and graded under microscope at low magnification and then total number of oocytes harvested was counted. The COCs were classified into 4 grades on the basis of cumulus cell attachment as described by Khandoker et al. (2001), briefly; Grade A: oocytes completely surrounded by cumulus cells; Grade B: oocytes partially surrounded by cumulus cells; Grade C: oocytes not surrounded by cumulus cells and Grade D: degeneration observed both in oocytes and cumulus cells. The grade A and B were considered as normal and grade C and D as abnormal COCs. The number of different grades of COCs in each category was recorded.

### 2.3 *In vitro* maturation of COCs

The maturation medium, tissue culture medium-199 (TCM-199) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with different levels of bovine serum albumin (BSA) (Sigma Chemicals Co. St. Louis, MO, USA) as 0.0% (Control), 2.5, 5.0 and 7.5% were prepared. The pH of all media was adjusted to 7.8 on the day of oocyte collection. From each group, about

2.5-3.5 mL of the medium was poured into 35 mm culture dishes. Then 1-4 drops (depending on number of oocytes) of 100  $\mu$ L of medium were poured into culture dish and covered with paraffin oil (Loba Chemical Pvt. Ltd., India), then the culture dish were placed in maturation conditions for 27 h (38.5 °C with 5% CO<sub>2</sub> in air with 100% humidity). Data was collected from four replicates.

### 2.3.1 *In vitro* cumulus cell expansion of COCs

After 27 h of IVM, cumulus cell expansion of COCs were determined by the examination of three levels of cumulus expansion (Rahman et al., 2003) at 10 $\times$  magnification under microscope and the number of COCs classified on the basis of expansion rate of COCs was recorded.

### 2.3.2 *In vitro* nuclear maturation

For this purpose, half of the matured COCs from each drop was taken and denuded by repeated pipetting. Oocytes were fixed for 24–48 h in a mixture of acetic acid and alcohol (1:3) at room temperature, stained for 10 min with 1% (*w/v*) orcein in 45% glacial acetic acid and examined for evidence of different stages of maturation under inverted microscope (Olympus, Germany) at high magnification (100 $\times$ ) with emersion oil. The different stages of nuclear maturation examined based on chromosomal configuration were assigned to germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase-I (M-I) and metaphase-II (M-II). A chromosome configuration was designated as GV, when having a single large nucleus with uniformly distributed filamentous chromatin subsequently condensing to form a ring of condensed chromatin around the compact nucleus. In the GVBD category, the nucleolus and nuclear membrane had disappeared and chromosomes appeared as condensed and coiled up filaments. The metaphase-I stage was recognized by the appearance of paired chromosomes and bivalents, while in the metaphase-II emission of first polar body, resulting in the formation of haploid set of chromosomes in the oocytes. Finally, percentage of nuclear maturation was calculated.

## 2.4 *In vitro* fertilization

The fertilization medium, Brackett and Oliphant (BO) (Crozet et al., 1995) was prepared and its pH was adjusted to 7.8 on the day of use. Semen was collected from the Black Bengal breeding bucks under the Department of Animal Breeding and Genetics, BAU, Mymensingh. Fifty micro litters of raw semen were taken in 10 mL sterilized pipette and 3.0-4.2 mL (depending on the sperm concentration) of semen washing solution was added to adjust the sperm concentration to 25 $\times$ 10<sup>6</sup> mL<sup>-1</sup>. Then the semen with

washing solution was taken in a centrifuge tube and it was centrifuged at 800 rpm for 5 min at 30 °C. After 5 min, the top liquid portion was removed by the digital pipette. Then same amount of semen washing solution was added to the centrifuge tube. The same procedure was repeated twice and finally the sperm concentration was adjusted at 1 $\times$ 10<sup>6</sup> mL<sup>-1</sup> by adding semen dilution solution (BO+2% BSA). Then 1-4 insemination droplets (100  $\mu$ L) 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 3-4 h for pre-incubation. After 27 h of maturation, the remaining half of the matured COCs (other half was used for nuclear maturation) was proceed 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 h in incubator at 38.5 °C with 5% of CO<sub>2</sub> in humidified air. After 5 hours of incubation, all the COCs from each drop were denuded 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 (100 $\times$ ) with emersion oil to observe pronuclei (PN) formation as- (a) Oocyte with two PN-normal fertilization; (b) Oocyte with one PN- asynchronous PN development/parthenogenetic activation or one PN was obscured by lipid droplets and (c) Oocyte with more than two PN – polyspermia.

## 2.5 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 (SAS, 1998).

## 3 Results and Discussion

### 3.1 *In vitro* maturation of COCs

In this study, the collected COCs were matured in TCM-199 supplemented with different levels of bovine serum albumin (BSA) to find out the effect of BSA on *in vitro* maturation of goat oocytes. Initially cumulus cell expansion was observed and then the nuclear maturation of COCs was examined.



### 3.1.1 *In vitro* cumulus cell expansion of COCs

The results of the *in vitro* cumulus cell expansion of goat COCs cultured in TCM-199 media supplemented with different levels of BSA were presented in Table 1. The level-3 expansion is the indicator of true maturation (Rahman et al., 2003) and the highest expansion was found in 7.5% BSA supplementation (75.55%), followed by 5% BSA (73.87%), 2.5% BSA (61.68%) and 0% BSA (45.36%). All the differences were found to be significant compared control but the difference observed plateauing at 5% level of BSA (Table 1). As the cumulus cell expansion in level-3 increased (indication of maturation), the other expansion levels decreased, because a portion of COCs expanded in level-2 and 1 were reached to expansion level-3. The result of BSA supplementation (73.87% maturation rate) in IVM media at 5% level found in this experiment was similar with the maturation rate of oocytes using TCM-199 supplemented with estrus goat serum (Kharche et al., 2006) and with fetal calf serum (Mondal et al., 2008).

### 3.1.2 *In vitro* nuclear maturation

The result of *in vitro* nuclear maturation of COCs cultured in different levels of bovine serum albumin (BSA) is presented in Table 2. The percentages of COCs matured up to metaphase-II stage were 40.78, 57.74, 67.52 and 68.95; metaphase-I were 28.11, 20.13, 18.46 and 17.68; germinal vesicle breakdown were 10.50, 12.35, 9.23 and 8.91; germinal vesicle were 20.60, 4.79, 7.79 and 4.46 at 0% (control), 2.5%, 5.0% and 7.5% level of BSA, respectively (Table 2). Metaphase-II stage oocyte participate in fertilization process and the highest M-II was found in 7.5% BSA (68.95%) and followed by 5.0% BSA (67.52%), 2.5% BSA (57.74%) and 0% BSA (40.78%). All the BSA supplementation groups found significantly higher ( $p < 0.05$ ) than that of the control group (0% BSA). Though the increased supplementation of BSA gradually increased the M-II but M-II again reached on plateau at 5% BSA (Table 2). GV, GVBD and M-I are not the indicator of oocyte maturation. Similar level of BVBD was recorded in 0% to different level of BSA (Table 2). On the other hand, almost similar level of M-I were found in 2.5, 5.0 and 7.5% BSA supplementation (20.13, 18.46 and 17.68%, respectively) and significantly highest ( $p < 0.05$ ) M-I was found in control group (28.11%). This is unusual and the cause is not clear. The nuclear maturation rate at Metaphase-II stage (67.52%) of oocytes with 5% BSA supplementation in maturation media was comparable with the maturation rates (63.72%) obtained with 10% fetal bovine serum supplementation (Kharche et al., 2009), 65.62% with  $5.0 \mu\text{g mL}^{-1}$  FSH,  $5 \mu\text{g mL}^{-1}$  LH and  $1 \text{ ng mL}^{-1}$  oestradiol, 25 mM Hepes, 0.25 mM pyruvate and antibiotics supplementation (Garg and Purohit, 2018) and 67.24% with  $1 \text{ mg mL}^{-1}$  estradiol and antibiotics supplementation (Mar-

chal et al., 2003). The results obtained in this study showed that BSA supplementation had an important role on both of *in vitro* cumulus cell expansion and nuclear maturation of Black Bengal goat oocytes. Similar advantageous results of the serum supplementation were reported by other authors in several animal species such as cattle (Sanbuissho and Threlfall, 1990), porcine (Nakanishi et al., 1990), and equine (Campo et al., 1992). The trend of *in vitro* nuclear maturation was similar with the *in vitro* cumulus cell expansion of COCs, which strengthen that 5.0% BSA supplementation in maturation media was optimum for IVM of goat oocytes.

### 3.2 *In vitro* fertilization of oocytes

After maturation of COCs in TCM-199 supplemented with different levels of BSA, all of the COCs or partly were fertilized with fresh Black Bengal buck semen and the rates of pronuclei formation is summarized in Table 3. It was observed that significantly ( $p < 0.01$ ) higher percentage (35.52% and 37.74%) of normal fertilization (formation of 2 pronuclei) was observed in 5.0 and 7.5% BSA level compared to control (23.28%) and 2.5% level (29.30%). In this study, it was observed that the normal fertilization (2 PN formations) significantly ( $p < 0.01$ ) increased with BSA supplementation (2.5% level) compared to control (Table 3). The similar significant ( $p < 0.01$ ) trend was observed by increasing the BSA level to 5.0% level but no such significant ( $p > 0.05$ ) trend was found when comparison was made between 5.0% and 7.5% BSA supplementation (Table 3). Polyspermic fertilization was observed in case of 2.5% and 5.0% level of BSA supplementation at a rate of 2.38% and 4.31%, respectively but there was no significant ( $p > 0.05$ ) difference between them. Insignificant difference ( $p > 0.05$ ) was also observed in one pronuclei (1 PN) formation for different levels of BSA.

The fertilization rate is directly dependent on the maturation of oocytes. Thus the fertilization rates in this study showed a significant differences ( $p < 0.01$ ) between BSA supplemented (2.5%) and control and also between 2.5% and 5.0% level of BSA supplementation in maturation media (Table 2) but no significant difference ( $p > 0.05$ ) was found between the BSA level of 5% and 7.5% (Table 2). After comparing the fertilization rate among the groups of oocytes matured in TCM-199 supplemented with 2.5% BSA and non-supplemented (control) and among the levels of BSA supplemented groups, it can be suggested that 5.0% BSA supplementation in maturation media yielded better results on further fertilization. Although supplementation of BSA at 7.5% level resulted similar fertilization rate ( $p > 0.05$ ) to 5.0% level (35.52% vs. 37.74%), considering the economic point of view, bovine serum albumin at 5.0% level of supplementation is optimum than 7.5% level.

Table 1. *In vitro* cumulus cell expansion of COCs cultured in media supplemented with different levels of bovine serum albumin

BSA level †	Total no. of oocytes	Expansion level (%) (Mean ± SE) ‡		
		Level-1	Level-2	Level-3
0.0% (control)	137	28.65a ± 1.01 (42)	26.01a ± 0.56 (40)	45.36c ± 0.94 (55)
2.5%	141	17.35b ± 1.50 (34)	20.98b ± 0.66 (37)	61.68b ± 0.88 (70)
5.0%	148	9.18c ± 1.40 (28)	16.95c ± 1.13 (35)	73.87a ± 0.27 (85)
7.5%	150	10.00c ± 0.84 (29)	14.49c ± 1.20 (33)	75.55a ± 0.58 (88)

† BSA = Bovine serum albumin; ‡ Means with different letters within the same column differ significantly (p<0.05); figure in the parenthesis indicates the number of oocytes.

Table 2. *In vitro* nuclear maturation of COCs cultured in media supplemented with different levels of bovine serum albumin

BSA level †	Total no. §	Nuclear maturation rate (%) (Mean ± SE) ‡			
		GV	GVBD	M-I	M-II
0.0% (control)	79	20.60a ± 2.81 (18)	10.50 ± 3.09 (14)	28.11a ± 1.68 (21)	40.78c ± 3.84 (26)
2.5%	80	9.79b ± 1.78 (14)	12.35 ± 2.09 (15)	20.13b ± 2.78 (18)	57.74b ± 2.39 (33)
5.0%	83	4.79b ± 2.41 (12)	9.23 ± 2.07 (14)	18.46b ± 1.54 (18)	67.52a ± 0.85 (39)
7.5%	84	4.46b ± 2.25 (12)	8.91 ± 2.23 (14)	17.68b ± 1.73 (17)	68.95a ± 1.38 (41)

† BSA = Bovine serum albumin; § Total number of COCs cultured; ‡ Means with different letters within the same column differ significantly (p<0.05); figure in the parenthesis indicates the number of oocytes.

Table 3. *In vitro* fertilization of COCs based on pronuclei (PN) formation cultured in media supplemented with different levels of bovine serum albumin

BSA level †	Total no. §	Fertilization rate (%) (Mean ± SE) ‡			
		2 PN	1 PN	>2PN	No PN
0.0% (control)	68	23.28c ± 3.00 (19)	5.59 ± 2.83 (12)	0.00 (0)	71.13a ± 1.02 (37)
2.5%	76	29.30b ± 0.73 (22)	5.13 ± 5.13 (12)	2.38 ± 1.08 (11)	63.19a ± 5.11 (31)
5.0%	85	35.52a ± 1.21 (26)	11.29 ± 2.53 (15)	4.31 ± 2.16 (12)	48.89b ± 1.11 (32)
7.5%	74	37.74a ± 1.24 (27)	15.60 ± 2.26 (17)	0.00 (0)	46.67b ± 3.33 (30)

† BSA = Bovine serum albumin; § Total number of COCs fertilized; ‡ Means with different letters within the same column differ significantly (p<0.05); figure in the parenthesis indicates the number of oocytes.

The result of normal fertilization in this study was comparable to the observation of Mondal et al. (2008) who reported 27.78–38.23% fertilization rate in goat IVF in case of 5.0% BSA supplementation in maturation media. Rodriguez et al. (2001) reported that the percentage of goat oocytes with two pronuclei in IVF to be 21.0–39.7% which was also in accordance with the result presented here. From the previous findings, it is revealed that BSA has a nutritional role to play by supplementing amino acids after hydrolysis; thereby maintaining the intracellular amino acid pools (Biggers, 1997). BSA also provide undefined embryotrophic (e.g. citrate, steroids) compounds (Biggers, 1997; Bavister, 1995; Gray et al., 1992), 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, an increase in internal  $\text{Ca}^{2+}$ , pH and membrane hyperpolarization (Storey, 1995). 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 (Visconti et al., 1995). A positive correlation between cholesterol depletion of the sperm plasma membrane and improved capacitation of sperm has been obtained in vivo (Davis, 1982) and *in vitro* (Cross, 1996). The components that act as cholesterol receptors such as albumin are present in the female tract (Benoff et al., 1993b; Go and Wolf, 1985; Davis et al., 1980). Thus, albumin is one of the prominent proteins supporting *in vitro* capacitation by accepting cholesterol (Benoff et al., 1993a; Go and Wolf, 1985; Davis et al., 1980). It has been demonstrated by Tajik and Esfandabadi (2003) that the penetration of cumulus free oocytes in protein free media in bovine are significantly ( $p < 0.05$ ) lower, compared with BSA contained media.

#### 4 Conclusions

From the present study, it is revealed that cumulus cell expansion, nuclear maturation and fertilization rate of goat oocytes could be significantly increased by supplementing upto 5.0% level of BSA. Therefore, BSA at 5.0% level might be economically viable and be used as a supplement in the maturation media for the Black Bengal goat oocytes.

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#### Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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