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Subsequent Development of Bovine Oocyte on *In Vitro* Maturation of Culture Media

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Abstract **– The study was undertaken to find out the development of bovine oocyte supplementation by Bovine Serum Albumin (BSA) on *in vitro* maturation of culture media. Cumulus oocytes complexes (COCs) were collected from bovine ovaries by aspiration method and matured for 48 hours in TCM-199 basic medium supplemented with different levels of BSA (0%, 5%, 10% and 15%). The Significantly higher ($p<0.01$) number of follicles were aspirated per ovary in ovaries without CL (4.60 ± 0.28) than with CL containing ovaries (3.00 ± 0.37). Higher numbers of COCs were found in ovaries without CL (4.42 ± 0.30) than ovaries with CL (2.40 ± 0.40). Higher number of follicles aspirated ($p<0.01$) and the collected number of COCs in left ovaries (4.76 ± 0.20) and (4.47 ± 0.19 per ovary) respectively than right ovaries (4.74 ± 0.20) and (4.34 ± 0.18 per ovary) respectively. So, left ovary without CL is a good source of normal grade oocytes for *in vitro* maturation of bovine oocytes. The percentage of COCs reached to Metaphase-II stages with 0%, 5%, 10% and 15% of BSA supplementation were 0.00%, 0.00%, 0.12% and 0.00% respectively. These results further indicate that the maturation and subsequent development rate could be significantly increased ($p<0.01$) by supplementing 5% level of BSA. This trend maintained up to 10% of BSA, but no further improvement was found by increasing level of BSA up to 15%. It is conducted that BSA has a positive effect on *in vitro* maturation of bovine oocytes and a 10% level of BSA is recommended based on the improvements observed and the associated economic benefits.**

Keywords **– Bovine Oocyte, Corpus Luteum, Bovine Serum Albumin, *In-vitro* Maturation.**

I. INTRODUCTION

Latest developments in gametes and embryo cellular biology, the field of molecular embryology on farm animals has been explored due to the limited availability of the suitable experimental materials at a acceptable cost. After a dramatic development of cellular biology over the last decades, lots of research efforts have been moved towards the implementation of assisted reproductive technologies (ARTs) such as multiple ovulation and embryo transfer (MOET), *In-vitro* production (IVP) of embryos, cloning and transgenesis to transfer a targeted number of embryos produced from animal having desired genetic make-up. In MOET, embryos are collected in-*vivo* from super ovulated donors at the required developmental stage and transferred to a number of synchronized recipients. Cattles are numerically and economically very important and promising animal genetic resources in developing country like Bangladesh and accounted for about 24.3 million head (DLS-2020). Cattle significantly tribute to the GDP in Bangladesh through production of milk, meat and skin.

Due to lower genetic potentiality, indigenous livestock cannot fulfill the demand of milk and meat. This is being considered as an important problem for livestock development and this can be overcome through genetic improvement of indigenous stock by an appropriate breeding technology such as reproductive and molecular genetic technologies. Assisted reproductive technologies include artificial insemination (AI), multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embryo etc. By enhancing selection intensity and reducing generation interval these technologies could bring tremendous change in animal production system.

In vitro maturation of immature oocytes from ovaries at slaughter, followed by IVF and IVC the resulting zy-

-gotes has allowed extensive research on modern reproduction techniques in farm animals.

Bovine serum albumin (BSA) is a globular protein that is often used as a protein concentration standard in lab experiments as well as in numerous other biochemical applications. Derived from cows, BSA is extracted from cow blood using one of three different purification methods: cold-organic solvent fractionation, heat shock, and ion exchange chromatography.

However, a great deal of work has been done regarding collection of cumulus-oocyte-complexes (COCs) from slaughter house ovaries, grading of collected oocytes, IVM, IVF of the oocytes and IVC of the zygotes throughout the world. But in Bangladesh, no such work has no far been undertaken. Slaughter house ovaries can be an economic source of oocytes for IVM, IVF and IVC experiment. Embryos can be produced from ovaries of the cows that are usually being slaughtered in slaughter houses for meat purpose and the embryos thus produced can be transferred to the recipient cows. Moreover, recent advances in biotechnology have enables the researchers to produce cloned and genetically modified animals by manipulating *in vitro* produced embryos. The present research work has been undertaken for the first time in Bangladesh as a very preliminary approach to embryos produced. The further approach to conduct a trial for *in vitro* maturation experiment could not be undertaken because of unavailability of some necessary instrument and apparatus at our disposal. But then it is hoped that the work would be a base line for the future researchers who will attempt to make further contribution in this field of animal biotechnology.

In Bangladesh, many female cattle are slaughtered year-round in the slaughter house and at the home. Ovaries from slaughter house female can be an economic source of oocytes for IVM, IVF and IVC experiment. Embryos produced from oocytes collected from ovaries can be transferred to the recipient cows. Moreover, recent advances in biotechnology have enabled the researchers to produced cloned and genetically modified animal by manipulating in *vitro* produced embryos. The present research work has been undertaken in Bangladesh to embryo production from oocytes collected from slaughter house ovaries. Under these circumstances the main objectives of the present study are as follows:

1. To investigate oocyte recovery rate and grading procedures of cumulus-oocyte-complexes (COCs) obtained from slaughter house bovine ovaries.
2. To establish the relationship between ovarian condition and morphological quality of COCs.
3. To know the effect of bovine serum albumin on *in vitro* maturation of bovine oocytes.

II. MATERIALS AND METHODS

The experiment was conducted at the Animal Nutrition, Breeding and Genetics Laboratory under the department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka from January 2019 to December 2019.

Preparation of the Laboratory

Before starting the experiment, all the necessary equipment were properly installed and checked. If needed, these things repaired and/or reinstalled. Finally all equipment cleaned and sterilised with 70% alcohol. All the reusable, equipment were properly washed, dried, covered with aluminium foil, sterilised and finally kept in a cleaned and sterilised chamber until use. All the essential disposal equipment as well as media, chemicals and

reagents were made readily available before starting the experiment.

The list of recruitments is mentioned below-

Microscope	Sterilised beaker	Glass microscope
CO2 incubator	Sterilised measuring 100 ml cylinder	Distilled water
Laminar Air Flow Cabinet	Culture dishes of 35mm	Digital pipette
Ph meter	Pasture pipette	Bovine serum albumin (BSA)
Weighing balance	Petre dishes (90mm)	Paraffin oil
Disposal 10 ml syringes	Water bath	Measuring scale
Disposal 18 G needles	Sterilized test tube 10 ml	Scissors
Sterilised rubber gloves	Tissue culture media 199 (TCM-199) for maturation	Watch glass
		Forceps

Collection and Processing of Ovaries

Preparation for Ovary Collection

Physiological saline (0.9% NaCl) was prepared and sterilized in autoclave and stored in refrigerator for further use. On the day of collection, 1000 mg of zentamycin were added per litre of saline. The solution was warmed at 25 to 30ºC and kept in a thermos box to maintain this temperature during transporting the ovaries from slaughter house to laboratory. Dulbecco's phosphate buffered saline (D-PBS) solution was also prepared by adding one pack of PBS salt in one litre of distilled water. Then it was sterilized in autoclave and stored in refrigerator for further use.

Collection of Ovaries and Trimming

The ovaries were kept in collection vial containing 0.9% Physiological saline in a thermo flask at 25 to 30 ºC and transported to the laboratory within 2 to 3 hours of slaughter. The ovaries were then transferred to the sterilised petridishes containing same saline at 25 ºC. In the laboratory each ovary was trimmed to remove the surrounding tissue and overlying bursa. Each ovary was treated to three washing in D-PBS.

Evaluation of Ovary

Ovaries were evaluated on the basis of following measures-

Measurement of Weight, Length and Width

Individually right and left ovary and ovaries with CL and without CL was weighed in a digital balance. The length and width in cm of the right and left ovary and ovaries with CL and without CL were measured with the help measuring scale.

Counting of Follicles on the Surface of the Ovary

There are numerous follicles on the surface of the both ovaries. The number of visible follicles on the surface of different category of ovaries were counted and recorded.

Collection of Follicles by Intact Follicle Collection Method

Each ovary was incised with scissors to collect all the visible follicle within it. Then follicles were stored in a saline watch glass at room temperature. From each follicle the follicular material were harvested with the help of forceps and needle by blunt dissection on a sterilised culture dish (35 mm). The COCs were classified into 4 grades: Grade-A = COCs completely surrounded by cumulus cells; Grade-B = COCs partially surrounded by cumulus cells; Grade-C = Oocytes not surrounded by cumulus cell; Grade-D = Degeneration observed both cumulus cells and oocytes. Grade A and Grade B considered as normal COCs. Grade C and Grade D considered as abnormal COCs. The number of different grades of COCs in each category were recorded.

COCs Aspiration and Grading

The ovaries were brought to the laboratory and washed 2-3 times in saline solution at 30ºC. ovary was picked up in left hand. The 10 ml syringe was loaded with PBS (1-1.5 ml), and needle (19G) was put in the ovary parenchyma near the vesicular follicles (2 to 6 mm diameter) by right hand and all 2 to 6 mm diameter follicles were aspirated near the point at the same time. After aspirating the follicles from one ovary, the aspirated follicular materials were transferred slowly into a 90 mm petridish, avoiding damaged to the cumulus cells and the COCs were searched and graded under microscope at low magnification (4X). The COCs were classified into 4 grades as described previously. The numbers of different grades of COCs in each categories were recorded. In the meantime another petridish of D-PBS was prepared for pooling COCs and the COCs were picked up with an appropriate glass micropipette.

The tip diameter of the pipette was checked under the microscope to ensure COCs, which could be easily aspirated without damaging the cumulus cells. The glass micropipette were prepared slowly stretching the tip of pasture pipette above burners flame and COCs were washed 2-3 times into D-PBS.

In Vitro Maturation (IVM) of Bovine COCs

Preparation of Medium and Droplet Culture Dish

The maturation medium, TCM-199 supplemented with 0% (control), 5%, 10% and 15% bovine serum albumin (BSA) were prepared and its P^H was fixed at 7.4 on the day of aspiration and sterilized by filtration. About 2.5 to 3.5 ml of the medium was poured into each of two 35 mm culture dishes. In another culture dish 4 drops of each about 100 µl of maturation medium were poured and covered with paraffin oil. The above two culture dishes with evenly distributed medium and dish with droplet were kept in an incubator at 38.5 ºC with 5% CO_2 in air. The first two dishes could be used for maturation of the oocytes.

Macroscopic Observation of Cumulus Cell Expansion

Normal graded (grade A and B) oocytes were washed to 3 times separately in PBS and then transferred into the maturation medium (TCM-199+ 0%, 5%,10% and 15% BSA) and washed 2 to 3 times. Droplets containing normal graded oocytes were kept in a CO_2 incubator at 38.5 ºC with 5% CO_2 in air for 48 hours. The numbers of oocytes according to grade used for maturation and the times of initiation of maturation were recorded. After 48 hours of IVM, cumulus expansion was determined by three level same magnificent under microscope as 1: indicating no expansion of COCs; 2: indicating moderate expansion and 3: indicating marked expansion cells with a compact layer of choronara diata. The number of oocytes classified on the basis of expansion rate COCs

was recorded.

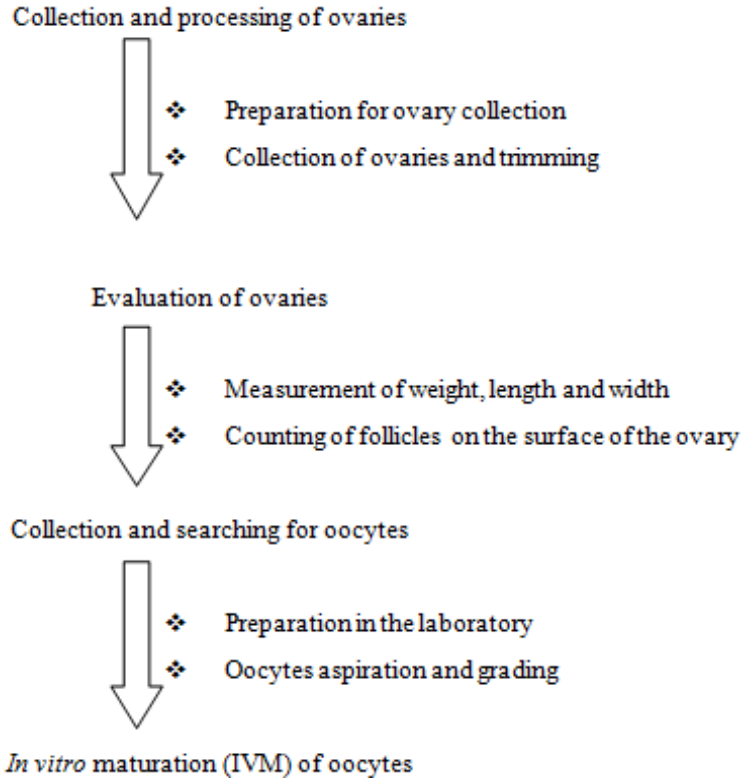


Fig. 1. Protocol of collection, evaluation and in vitro maturation of bovine oocyte.

Nuclear Maturation

The nuclear level maturation was checked in representation sample. For the purpose, after *in vitro* maturation for 48 hours. COCs were denuded from cumulus cells by repeated pipetting. Then oocytes were washed in PBS three times. After that the oocytes were transferred to Na-Citrate solution for 5-10 minutes. Oocytes were mounted on a glass slide and washed with aceto-alcohol up to complete removal of cytoplasm from the oocytes. Then it was stained with 1% aceto-orcein for 30 minutes. After drying, the slides were examined under inverted microscope at high magnification(100X) with immersion oil through USB 2.0 camera for germinal vesicle break down (GVBD), Metaphase-I (M-I) and Metaphase-II (M-II) stage. Finally, percentage of maturation was calculated.

Statistical Model and Methods of Data Analysis

All values were expressed as Mean ±SE. Comparison of means Duncan's multiple range test (DMRT) was applied with the help of statistical analysis system (SAS,1998).

III. Results and Discussion

Ovarian Classification (Left and Right) and Other Parameters Per Ovary

From local slaughter house cow ovaries were collected and were recorded as right and left. On the basis of presence and absence of corpus luteum (CL) again they were grouped as with CL and without CL. Among 70 ovaries CL found in 25 ovaries and remaining 45 ovaries having no CL. The result of the different parameters is summarized in table 1, 2, 3 and 4.

Table 1. Ovarian Classification (Left and Right) and other parameters per ovary.

Ovarian Type	Weight (g) (Mean ± SE)	Length (cm) (Mean ± SE)	Width (cm) (Mean ± SE)	Total No of Visible Follicle (Mean±SE)	No of Follicle Aspirated (Mean ±SE)	Collected COCs per ovary (Mean±SE)				
						Total	Normal		Abnormal	
							A	B	C	D
Right (70)	1.14±0.26	1.18^{b}±0.04	0.86 ±0.03	6.84±0.26 (176)	4.74±0.20 (113)	4.34±0.18 (76)	2.11^{a} ± 0.09 (23)	1.52^{a} ± 0.08 (24)	0.33^{b} ± 0.09 (6)	0.37^{b} ± 0.09 (23)
Left (68)	1.13±0.26	1.31^{a}±0.04	0.90 ±0.03	6.51±0.27 (198)	4.76±0.20 (121)	4.47 ± 0.19 (86)	0.37^{b} ± 0.09 (17)	0.24^{b} ± 0.08 (10)	1.68^{a} ± 0.09 (21)	2.19^{a} ± 0.09 (38)
Significant Level	NS	**	NS	NS	NS	NS	**	**	**	**

Means with different superscripts differ significantly from each other within the same column ** ($p<0.01$). NS = Not significant.

Parenthesis indicates the total number.

Among 138 ovaries (consisting right ovary 70 and left ovary 68) a number of 45 belonged without CL and 25 with CL. The result of different parameters is summarized in Table 1 and Table 2. The length (cm) of left (1.31 ± 0.04) was significantly ($p<0.01$) higher than the right ovary (1.18 ± 0.06) but no significant differences were found in the width (cm) and weight (g) of right ovaries (0.86 ± 0.03 and 1.14 ± 0.26) and left ovaries (0.90±0.03 and 1.13 ± 0.26) respectively (Table 1). The number of 374 follicles were recorded on the surface of the ovaries and 234 follicles were aspirated from the surface of both (right and left). Ovaries among them 113 were obtained with a mean of (4.74 ± 0.20) per ovary from right and 121 from left ovaries with a mean of (4.76 ± 0.20) per ovary (Table 1). The collected number of COCs higher in left ovaries (4.47 ± 0.19) compared to right ovaries (4.34 ± 0.18). When the COCs were classified as normal and abnormal groups, the highest number of normal COCs were found in right (Grade-A; 2.11 ± 0.09 and Grade-B; 1.52 ± 0.08) than that of left (Grade-A; 0.37 ± 0.09 and Grade-B; 0.24 ± 0.08) ovary. Age, season, nutritional status (body condition) and cyclicity of animals at the time of slaughter, size and functional status of follicles, methods of oocyte retrieval are some of the factors that might contribute to recorded variation in oocyte quality (Nandi et al., 2001; Zoheir et al., 2007; Amer et al., 2008). A number of research works have been conducted to compare the efficiency of the oocyte collection techniques in cattle (Katska, 1984; Lonergan et al., 1991), sheep (Wahid et al., 1992; Wani et al., 2000) and goat (Mogas et al., 1992; Wang et al., 2007). In Bangladesh, few researches have performed in IVP of goat embryos, where COCs were collected only by aspiration of 2 to 6 mm diameter follicles (Asad et al., 2016; Islam et al., 2007; Mondal et al., 2008; Ferdous, 2006).

Table 2. Ovarian classification (with CL and without CL) and other parameters per ovary.

Ovarian Type	Weight (g) (Mean ±SE)	Length (cm) (Mean±SE)	Width (cm) (Mean±SE)	Total No of Follicle (Mean ± SE)	No of Follicle Visible Aspirated (Mean ± SE)	Collected COCs per ovary (Mean ± SE)				
						Total	Normal		Abnormal	
							A	B	C	D
With CL (25)	5.14^{a} ± 0.21	2.75^{a} ± 0.08	2.26^{a} ± 0.06	4.32^{b} ± 0.43 (108)	3.00^{b} ± 0.37 (75)	2.40^{b} ± 0.40 (60)	0.40^{b} ± 0.21 (10)	0.24^{b} ± 0.14 (6)	0.24 ± 0.12 (6)	1.52^{a} ± 0.15 (38)

Ovarian Type	Weight (g) (Mean ±SE)	Length (cm) (Mean±SE)	Width (cm) (Mean±SE)	Total No of Follicle (Mean ± SE)	No of Follicle Visible Aspirated (Mean ± SE)	Collected COCs per ovary (Mean ± SE)				
						Total	Normal		Abnormal	
							A	B	C	D
Without CL (45)	$3.28^{b} \pm 0.15$	$2.27^{b} \pm 0.06$	$1.68^{b} \pm 0.05$	$5.93^{a} \pm 0.32$ (267)	$4.60^{a} \pm 0.28$ (207)	$4.42^{a} \pm 0.30$ (199)	$2.26^{a} \pm 0.16$ (102)	$0.75^{a} \pm 0.11$ (34)	0.48 ± 0.09 (22)	$0.91^{b} \pm 0.11$ (41)
Significant level	**	**	**	**	**	**	**	**	NS	**

Means with different superscripts differ significantly from each other within the same column** ($p<0.01$), NS= Not significant. Parenthesis indicates the total number.

In other case 282 follicles were aspirated out of 375 follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase). The width, length and weight were significantly ($p<0.01$) higher in ovaries with CL ($5.14^{a}\pm0.21$, $2.75^{a}\pm0.08$ and 2.26^{a} ±0.06) than those of ovaries without CL ($3.28^{b}\pm0.15$, $2.27^{b}\pm0.06$ and $1.68^{b}\pm$ 0.05) (Table-2). The Significantly higher ($p<0.01$) number of follicles were aspirated per ovary in ovaries without CL (4.60±0.28) than in CL containing ovaries (3.00±0.37) (Table 2). The CL is an extra cellular material within the ovary which made differences of its width and weight (Jablonka-Sharif *et al.*,1993).This result contradicts with the previous result of Singh *et al*.,(1994), Rahman *et al*.,(1977) and Sanker *et al*., (1993) and it might be due to less number of ovaries were processed.

Higher numbers of COCs were found in ovaries without CL (4.42±0.30) than ovaries with CL (2.40±0.40). Furthermore, COCs were classified in normal and abnormal groups, the significantly higher ($p<0.01$) number of normal COCs was found in ovaries without CL (Grade-A;2.26±0.16 and Grade-B; 0.75±0.11) than those ovaries with CL (Grade-A;0.40±0.21 and Grade-B; 0.24±0.14) and the reverse trend was found in abnormal group (Grade-C;0.48±0.09 and Grade-D; 0.91±0.11) and (Grade-C; 0.24±0.12 and Grade-D; 1.52±0.15) follicles per ovary respectively. When compared the ovaries in between with CL and without CL group, significantly ($p<0.01$) higher number of normal COCs were found in without CL group than that of with CL group. The result strongly supported by the previous finding of Asad (2015) who reported that higher number of follicles aspirated per ovary without CL group (2.92±0.08) than those of the with CL group (2.52±0.11) in goat. Similarly, finding also found in buffalo ovaries by Khandoker *et al.*, (2011) where significantly higher number of follicles were collected in ovaries without CL (6.78±0.18) than in CL containing ovaries (4.09±0.26). Similar results also reported in goat (Saha *et al*., 2014; Mondal *et al*., 2008 and Islam *et al*., 2007). In case of cows, ovaries having without CL contributing more total number of COCs per ovary (6.8±1.0) and also contributing higher normal COCs (5.7±0.9) than that of ovaries with CL (6.0±2.0 and 4.5±1.5 respectively) (Khandoker *et al*., 2016).

The follicular growth is inhibited while atresia is increased in presence of CL in the ovary (Hafez, 1993). The presence of CL in cyclic female's ovary produces a higher level of progesterone hormones that signals a negative response to anterior pituitary gland for the restriction of gonadotrophin secretion and ultimately follicular degeneration oocurs (Webb *et al*.,1999). In this study, the average number of collected COCs per

ovary were significantly higher ($p<0.01$) in CL-absent ovaries due to the absence of corpus luteum in non-cyclic female. Nandi *et al*., (2000) stated that when ovaries has corpus luteum, the oocytes recovery rate decreases. This is because there will be restriction of follicular development as lutein cells occupy most of the ovary (Kumar *et al*., 2004). Within the category, the highest number of normal COCs than that of abnormal COCs further supports the above statement (Khandoker *et al*., 2011; Asad, 2015) who found that presence of a CL significantly reduced the recovery rate as well as the quality of the oocytes. These statement can be the physiological explanation for lower number of COCs in the CL ovaries compared to without CL ovaries. Our finding further supported by other researchers, they have done their research in goats (Asad, 2015; Khandoker *et al*., 2011; Mondal *et al*., 2008 and Islam *et al*., 2007).

In Vitro Maturation (IVM) of COCs

In vitro maturation of COCs can be divided into nuclear and cytoplasmic processes. Nuclear maturation involves resumption of meiosis and progression to metaphase II. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed in order for the embryo to be fertilized and develop into a normal embryo and offspring (Eppig, 1996). In this experiment, only normal quality of COCs (Grade-A and Grade-B) collected by aspiration technique were taken and similar media and condition were used for aspiration technique for maturation and further processed to fertilization. The maturation of COCs was initially measured by macroscopic observation of cumulus cell expansion level and then confirmed by nuclear maturation.

a) *Cumulus Cell Expansion of COCs after 48 h Culture*

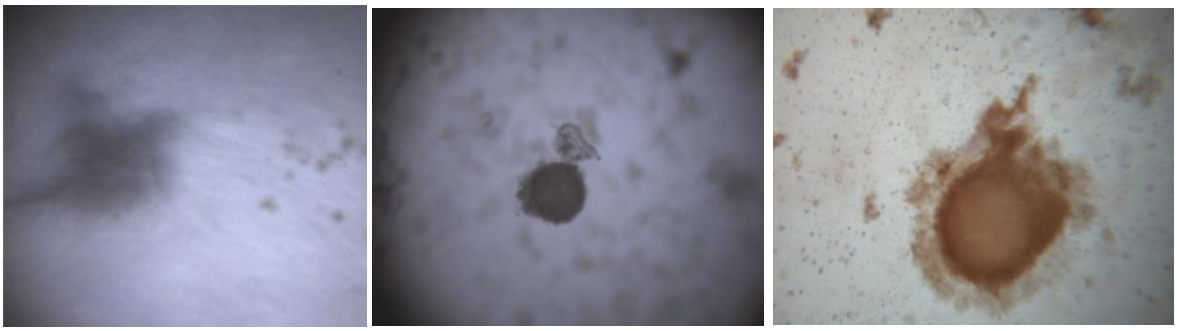
The result of the cumulus cell expansion of COCs cultured in TCM-199 supplemented with different levels of Bovine Serum Albumin (BSA) are presented in Table 3. No significant difference ($p>0.05$) was found in level-1 but highest value was found when TCM was supplemented with 15%BSA ($0.50^{a}\pm0.13$). In level-2, highest value was found when TCM is supplemented with 10% BSA ($0.86^{a}\pm0.15$) and significant difference ($p<0.05$) were observed when TCM was with 0%, 5%, 10% and 15% BSA ($0.38^{ab}\pm0.17$, $0.43^{ab}\pm0.14$ and $0.31^{b}\pm0.17$). Incase of Level-3, highest value was observed ($0.96^{a}\pm0.13$) when TCM was supplemented with 5% BSA but no improvement was observed when TCM was increased at 15% level ($0.06^{b}\pm0.16$) BSA.

Table 3. In-Vitro-Maturation of COCs after 48 hours of culture in different category of ovaries.

Level of Bovine Serum Albumin (BSA)	**Total No of Normal Oocytes (COCs)**	**Rate of Expansion level % (mean±SE)**		
		Level-1	**Level-2**	**Level-3**
TCM+0%BSA (Without supplementation)	16	0.32 ± 0.13	$0.38^{ab} \pm 0.17$	$0.13^{b} \pm 0.16$
TCM+5%BSA	23	0.26 ± 0.11	$0.43^{ab} \pm 0.14$	$0.96^{a} \pm 0.13$
TCM+10%BSA	21	0.29 ± 0.11	$0.86^{a} \pm 0.15$	$0.71^{a} \pm 0.14$
TCM+15%BSA	16	0.50 ± 0.13	$0.31^{b} \pm 0.17$	$0.06^{b} \pm 0.16$
Significant level		NS	*	*

Values are shown in Mean±SE. Means with different superscripts within the column* differ significantly ($p<0.05$). NS = Not significant.

The result of cumulus cell expansion are also demonstrated in plate-1, which clearly indicated the differences of expansion level of COCs under different BSA supplementation.



A B C

Plate 1: Photograph of different level of cumulus cell expansion.

Where;

A. Cumulus cell expansion level-1 (less expansion)

B. Cumulus cell expansion level-2 (moderate expansion)

C. Cumulus cell expansion level-3 (marked expansion)

b) *In-Vitro Nuclear Maturation*

The result of nuclear maturation of COCs after 48 hours cultured in different levels of BSA is presented in Table 4. The percentage of COCs matured up to metaphase II stage were 0.00%, 0.00%, 0.12% and 0.00%; metaphase-I were 0.00%, 0.92%,0.47%, and 0.28% ; GVBD were 0.64%, 0.62%,0.82% and 0.61% and GV 0.29%, 0.19%, 0.24% and 0.44% for without (control), 5%, 10%,15% level of BSA respectively (Table 4).

Table 4. Effect of different levels of BSA on in vitro nuclear maturation Of COCs.

Level of Bovine Serum Albumin (BSA)	**Total No of Normal Oocytes (COCs)**	**Rate of nuclear maturation % (Mean± SE)**			
		M-I	**M- II**	**GVBD**	**GV**
TCM+0%BSA (without supplementation)	14	$0.00^{c} \pm 0.17$	0.00 ± 0.04	0.64 ± 0.20	0.29 ± 0.14
TCM+5%BSA	21	$0.92^{a} \pm 0.14$	0.00 ± 0.03	0.62 ± 0.16	0.19 ± 0.11
TCM+10%BSA	17	$0.47^{b} \pm 0.15$	0.12 ± 0.04	0.82 ± 0.18	0.24 ± 0.13
TCM+15%BSA	18	$0.28^{bc} \pm 0.15$	0.00 ± 0.03	0.61 ± 0.17	0.44 ± 0.12
Significant level		*	NS	NS	NS

Values are shown in Mean±SE. Means with different superscripts within the column*differ significantly($p<0.05$).

NS = Not significant. M-I = Metaphase- I, M-II = Metaphase- II. GVBD = Germinal vesical break down. GV = Germinal vesicle.

In this study, significant difference ($P<0.05$) was found in the oocytes classified as M-II stages between Bovine Serum Albumin supplemented 5% and 10% and between 0% (control) and 5% level of Bovine Serum Albumin but no significant difference ($p>0.05$) found and the level of Bovine Serum Albumin decreased from 10% to 15%. No difference was found in metaphase-II but the highest M-II was found in 10% BSA (0.12%) supplementation. Significant difference ($p<0.05$) was found in metaphase-I and highest value was found in 5% BSA (0.92a ± 0.14). No significant difference was found in GVBD and GV but highest was in 10% BSA (0.82a

± 0.18) and 15% BSA (0.44a ± 0.12) respectively (Table-4 and plate-2). Holm et al., (1999) started that BSA is essential during in vitro maturation of bovine oocytes. Moreover, BSA was used with culture media as a source of protein by Rose and Bavister (1992). Three level of cumulus cell expansion after 24 h of in vitro maturation (at 38.5ºC and 5%CO2 in an incubatior) observed under 10x magnification of microscope and metaphase-II stages were 40.78 ± 3.84, 67.52 ± 0.85, 68.95 ± 1.88 and 57.74 ± 2.39% at 0, 2, 4 and 6 mg/ml of BSA supplementation on goat embryo (Asad et al., 2017).

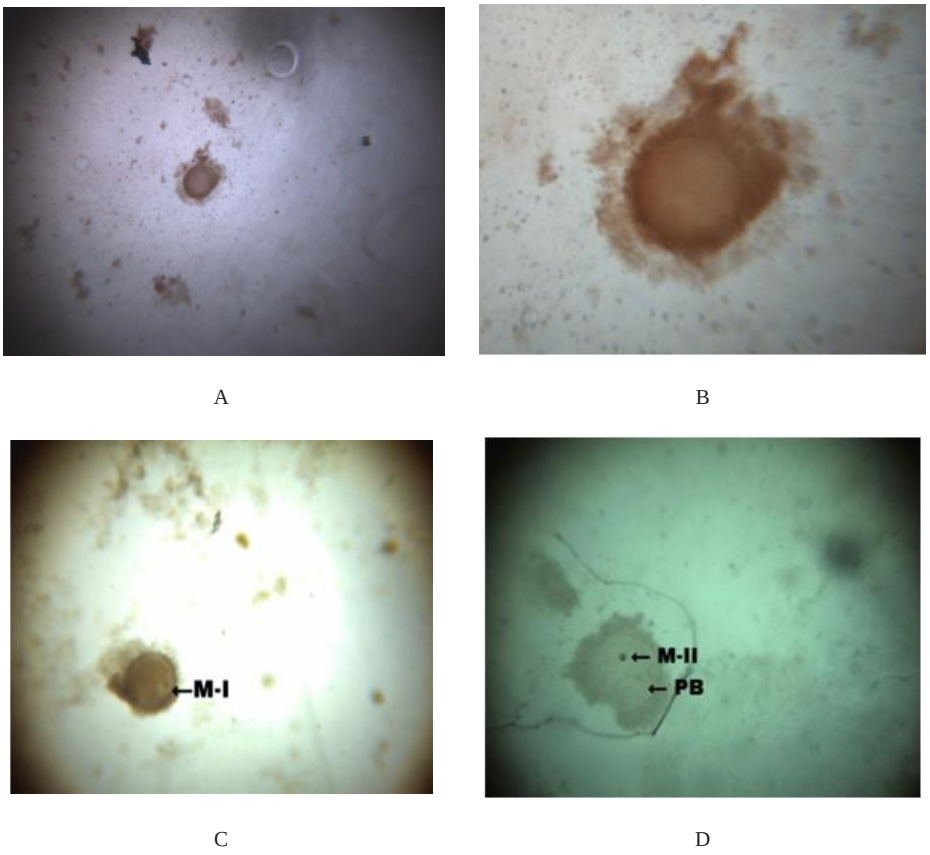


Plate 2. Photograph showing different stages of nuclear maturation of COCs based on chromosomal configuration.

Where,

A. Germinal vesicle (GV)

B. Germinal vesicle breakdown (GVBD)

C. Metaphase- (M-I) indicated by arrow and

D. Metaphase- (M-II) indicated by arrow and PB indicates polar body.

The nuclear maturation rate at M-II (0.12%) of oocytes using 10% BSA supplementation in maturation media was comparable with the maturation rates obtained with 10% fetal bovine serum supplemented (63.7%; Kharche *et al.*,2006), 10-20% estrus goat serum (58-71%; Kharche *et al.*,2009) and with 10% fetal calf serum (58.8-60.4%; Wang *et al.*, 2007). The maturation rate was also comparable to the results of Asad *et al.*, (2018) who obtained the COCs reached to M- II at 10% level of FF (Follicular Fluid) supplementation was 66.66 ±0.00% in goat embryo and Wang *et al*. (2007) who obtained 48-63% maturation rate in Boer goat. The maturation rate was also comparable to the result of Hoque (2009) who obtained 58.7% maturation rate in Black Bengal Goat.

The findings of the present study were comparable with those of cattle (Choi *et al*. 1997 and Caralon *et al*.1992); buffalo (Asad *et al*.,2012 and Chauhan *et al*., 1997); sheep (Wani *et al*., 2000) and also with goats (Pawshe *et al*.,1994).

In the present study, the rate of overall maturation is 0.12% contrasting to the finding, the *in vitro* maturation rate of oocytes of zebu cows was higher in Bangladesh (Das *et al*., 2006; Talukder *et al*., 2008). Moreover, grades of oocytes may influence the *in vitro* maturation rates of oocytes as variation in rate of maturation *in vitro* was demonstrated between good and poor grade oocytes (Goswani 2002). However, all retrieved oocytes were cultured for maturation irrespection of grading which may contribute for obtaining lower maturation rate in the present study than earlier.

The present study focused on the influence of different level of BSA supplementation on IVM of bovine oocytes. After the above discussion, we could conclude that, considering the effects of BSA, on the *in vitro* maturation of bovine, 10 % BSA level can be advantageous as a supplementation of maturation. Moreover, this result creates a great opportunity of conducting further research on bovine embryo production.

IV. Conclusion

The research work was conducted at the Animal Nutrition, Genetics and Breeding Laboratory Department of Animal Nutrition, Genetics and Breeding, Sher-E-Bangla Agricultural University, DHAKA-1207 with a view to establish the suitable method of oocyte collection, evaluation of slaughterhouse bovine ovaries and COCs depending on some parameters to establish the procedure of in *vitro* nuclear maturation of bovine oocyte and also the culture condition of IVM. The objective of research work was to find out the effect of bovine serum albumin on *in-vitro* maturation of bovine oocytes.

In this research, 282 follicles were aspirated out of 375 follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase). The Significantly higher ($p<0.01$) number of follicles were aspirated per ovary in ovaries without CL (4.60 ± 0.28) than in CL containing ovaries (3.00 ± 0.37). Higher numbers of COCs were found in ovaries without CL (4.42 ± 0.30) than ovaries with CL (2.40 ± 0.40), the significantly higher ($p<0.01$) number of normal COCs was found in ovaries without CL than those ovaries with CL with the mean of (Grade-A; 2.26 ± 0.16 and Grade-B; 0.75 ± 0.11) and (Grade-A; 0.40 ± 0.21 and Grade-B; 0.24±0.14) follicles per ovary respectively. Number of follicles aspirated in left ovaries (4.76±0.20) and in right ovaries (4.74 ± 0.20). The collected number of COCs higher in left ovaries (4.47 ± 0.19 per ovary) compared to right ovaries (4.34 ± 0.18 per ovary). So, left ovary without CL is a good source of normal grade oocytes for *in vitro* maturation of bovine oocytes.

In case of bovine serum albumin (BSA) supplementation in IVM media, it was found that the percentage of COCs reaching the cumulus cell expansion level-3 were 0.13 ± 0.16, 0.96 ± 0.13, 0.71 ± 0.14, 0.06 ± 0.16; M-II stages were 0.00 ± 0.04, 0.00 ± 0.03, 0.12 ± 0.04, 0.00 ± 0.03 with 0% (control), 5%, 10% and 15% of BSA supplementation. Where, 5% BSA supplementation was reached 0.96% at cumulus cell expansion level-3 and nuclear maturation at 10% BSA reached 0.12% M-II. The result of this experiment indicates that10% BSA level could be used as a supplement in TCM-199 maturation media.

Finally, it can be conducted that, left ovaries contain more COCs and higher number of follicles than right ovaries; without CL group ovaries with 10% level of bovine serum albumin supplementation are suitable for *in*

vitro nuclear maturation of bovine oocytes.

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