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Effect of Follicular Fluid on in Vitro Maturation, Fertilization and Development of Goat Embryos using Fresh Semen

L. Asad[1*], M.A.M.Y. Khandoker[2], S.A.M. Hoque[3] and S. Saha[4]
[1]Associate Professor, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh
[2]Professor, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh
[3]Assistant Professor, Department of Animal Breeding and Genetics, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh
[4]Assistant Professor, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, Bangladesh
*Corresponding author e-mail: lamyea_sau@yahoo.com ; Phone: 00-88-01716902084 ; Fax: 00-88-8155800

***Abstract* – The study was undertaken to find out the beneficial effect of goat follicular fluid (FF) on *in vitro* maturation, fertilization using fresh semen and to support the subsequent goat embryo development. Cumulus oocytes complexes (COCs) were collected from goat ovaries by aspiration method and matured for 24 hours in TCM-199 basic medium supplemented with different levels of FF (5%, 10% and 15%). The percentages of COCs reached to Metaphase-II stages with 0%, 5%, 10% and 15% of FF supplementation were 43.33 ± 3.33, 51.67 ± 0.83, 66.66 ± 0.00 and 67.79 ± 1.92% respectively. The normal fertilization (formation of two pronuclei) was recorded as 27.47 ± 0.55, 31.69 ± 1.09, 36.54 ± 1.73 and 38.69 ± 2.95%, respectively. After fertilization the rate of development to compact morula was found as 7.95 ± 1.35, 10.17 ± 0.17, 13.34 ± 3.33 and 12.63 ± 1.33%, respectively. These results further indicate that the maturation, fertilization and subsequent development rate could be significantly increased (*p*<0.01) by supplementing 5% level of FF than that of control. This trend maintained up to 10% of FF, but no further improvement was found by increasing the level of FF up to 15%. Thus, this study suggests that follicular fluid supplementation at 10% level could be beneficial for maturation and culture of goat oocytes and embryos.**

***Keywords* – Black Bengal Goat, Goat Follicular Fluid, In-*Vitro* Culture, In-*Vitro* Fertilization and In-*Vitro* Maturation.**

I. Introduction

In vitro production of embryos refers to the use of laboratory technique to produce embryos. This process usually includes retrieval of oocytes from the ovaries of a female, *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of presumptive zygotes to the morula or blastocyst stage of embryo development. The maturation media and supplementation plays a vital role on potential development of oocytes in IVP procedure. Generally buffered Tissue Culture Medium-199 (TCM-199) is used as a basic medium for IVM of goat oocytes. To establish a well-defined medium, scientists added different supplements to this basic medium from different sources maintaining different levels [17].

Goat follicular fluid is one of the alternatives of macromolecules that can be used as the supplementation in the maturation media because it is easy to get and cheap. Follicular fluid, a liquid, is found in the follicular antrum and surrounded the ovum in an ovarian follicle. Follicular fluid is reported to consist of various growth factors [11], follicle stimulating hormone (FSH), luteinizing hormone, and several other nutrients [2]. The follicular fluid provides such environment where oocytes could nourish and undergo maturation like *in vivo*. Moreover, FF protects the oocyte from the factors that include the premature resumption of meiosis, guard the oocyte against proteolytic attack and facilitate its extrusion during ovulation and enhance spermatozoa attraction, motility and acrosome reaction [3]. A variety of research works have been conducted on the effect of follicular fluid supplementation on IVM in bovine, pig, buffalo and ovine, where Follicular fluid is being successfully incorporated in IVM media of cattle, equines and goats [1, 4, 5, 8, 15, 16]. However, the specific role of FF is still obscure. FF might have the ability of oocytes nuclear and cytoplasmic maturation [6]. As a by-product, after necessary treatments, follicular fluid can be used as a supplement in the maturation media (TCM-199) because fluid from normal follicle contains a balanced proportion of estradiol/ progesterone (EPR) and insulin-like growth factor binding proteins (IGFBPs) which enhance the maturation and fertilization of oocyte [15].

In Bangladesh, limited researches have been conducted on IVP of goat embryos where TCM-199 has been supplemented with 5% fetal calf serum (FCS) [14]. Therefore, the present research was undertaken to study the effect of follicular fluid supplementation in maturation, fertilization, and culture media for *in vitro* maturation, fertilization and subsequent development of goat embryos.

II. Materials and Methods

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

Collection and Evaluation of Ovaries and Oocytes

Goat ovaries were collected from local slaughterhouse. The ovaries were kept in collection vial containing 0.9% physiological saline in a thermo flask at 25^0C to 30^0C and transported to the laboratory within 4 to 5 hours of slaughter and ovary was treated to three washings in D-

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PBS and two washings in oocyte harvesting medium (DPBS + 4 mg/ml BSA + 1.50 IU/ml Penicillin) as described by Wani [18]. The oocytes with cumulus cell layer (generally termed as cumulus oocyte complexes COCs) were aspirated from 2-6 mm diameter surface follicles with a 18 G needle attached to a 10 ml syringe which was loaded with D-PBS (1.0-1.5ml) as described by Hoque [7]. The collected COCs were classified into 4 grades on the basis of cumulus cells and nucleus as described by Khandoker [9]. The grades were; 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. Grade A and B considered as normal and grade C and D as abnormal COCs.

Follicular Fluid Collection and Preparation

After necessary trimming and washing of ovaries, follicular fluid was collected from morphologically healthy follicles by aspiration method using 10 ml syringe with 19 G needle. Criteria for assessment of follicular health established by Kruip and Dieleman [12] for bovine ovaries were applied in this experiment to assess goat follicles briefly: Non-atretic: Uniform bright appearance, extensive and very fine vascularization and no free-floating particles in the follicular fluid. Atretic: Loss of translucency, slightly or dull grayish and/or opaque appearance and free-floating particles in follicular fluid. At each collection, fluid from each surface follicle was pooled, centrifuged at 3000 rpm for 30 minutes at 4^0C. The top portion liquids were collected and again centrifuged for 15 minutes at same rpm and temperature. The supernatant was collected and filtered through a 22µ millipore filter and then transferred into a sterile glass beaker for heat inactivation at 56^0C for 1 hour in a water bath and were stored in a deep freeze at -20^0C for subsequent use for (IVM).

In Vitro Maturation (IVM) of COCS

The maturation medium, TCM-199 (Sigma Chemical Co., USA) was prepared and divided in to 4 groups and they were supplemented with gFF at the rate of without (control), 5%, 10% and 15%, respectively. The pH of all media was adjusted to 7.4 on the day of oocyte collection and sterilized by passage through a 20 µm filter. From each group, about 2.5-3.5 ml of the medium was poured into each of two 35 mm culture dishes. This culture dishes could be used for washing of COCs then 1-4 drops (depending on number of oocytes) of 100 µl of medium were poured in another culture dish and the COCs were transferred to the droplets and covered with paraffin oil (Labo America, Inc., California, USA). Finally the dishes with droplet were kept in an incubator (38.50 C, 5% CO_2 in air, 90-95 % relative humidity). The first 2 dishes from each group could be used for maturation of the COCs. 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 immersion oil in computer screen through USB 2.0 camera for germinal vesicle (GV), metaphase-I (M-I) and metaphase-II (M-II) stage. Finally percentage of maturation was calculated.

In Vitro Fertilization (IVF) with Fresh Semen

The fertilization medium, Brackett and Oliphant (BO) was prepared and its pH was adjusted to 7.8 on the day of use. Finally it was sterilized by passing through a 20 µm Sartorius Minisart filter. Semen was collected by artificial vagina (AV) method from the bucks of USDA funded Black Bengal goat project, Department of Animal Breeding and Genetics, BAU, Mymensingh, and brought to the laboratory in icebox (at 4-5°C) within a short period and the sperm concentration of raw semen was calculated by haemocytometer. 50 µl of raw semen was taken in 10 ml sterilized pipette and 3.0-4.2 ml (depending on the sperm concentration) of semen washing solution (Brackett and Oliphant medium 50 ml and caffeine 0.1942 g) was added to adjust the sperm concentration to $2x10^6$ per ml. Then the semen was centrifuged at 800 rpm for 5 minutes at 30^0C. After 5 minutes, the top liquid portion was removed and 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 10^6 per ml by adding semen dilution solution (Brackett and Oliphant + 2% BSA). Then 1-4 insemination droplets (100 µl) of Brackett and Oliphant 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 hours for preincubation. After 24 hours of maturation, the half of the matured COCs was proceed to fertilization and other half was used for nuclear maturation test. 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 hours in incubator at 38.5^0C with 5% of CO_2 in humidified air.

After 5 hours of incubation, 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 under inverted microscope at high magnification (100 x) to observe pronuclei (PN) formation as - Oocyte with two PN – normal fertilization, Oocyte with one PN – asynchronous PN development/parthenogenetic activation or one PN was obscured by lipid droplets and oocyte with more than two PN – polyspermia. Finally the rate of fertilization was calculated.

In Vitro Culture (IVC) and Observation of Embryo Development

After 5 hours incubation, 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 FF. The dish was then kept in the CO_2 incubator at 38.5^0C of 5%

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CO_2 in air. The development was checked every 48 hours and the culture were continued for 6 to 7 days. The number of 2-cell, 8-cell and compact morula was recorded.

Statistical Analysis

All values were presented in tables in the form of mean ± SE. Comparison of the mean between different parameters, maturation, fertilization and rate of development was evaluated by using F test. The mean differences among the treatments were adjudged as per Duncan's Multiple Range Test. All statistical analyses were done by using Statistical Package for Social Science (SPSS) Package Computer Program.

III. Results

In Vitro Maturation of COCs

In this study, the collected COCs were matured in TCM-199 supplemented with different level of gFF to check the *in vitro* maturation of goat oocytes. The maturation of COCs was confirmed by checking the nuclear maturation. The result of *in vitro* nuclear maturation of COCs after 24 hour cultured in different levels of gFF is summarized in Table I and fig. 1. The percentages of COCs matured up to metaphase-II stage were 43.33, 51.67, 66.66 and 67.79%; metaphase-I were 23.31, 24.04, 16.66 and 16.10%; GVBD were 15.09, 11.29, 9.78 and 9.60%; GV were 18.28, 12.81, 6.61 and 6.42% for without (control), 5%, 10% and 15% level of gFF, respectively (Table I). The highest M-II was found in 15% gFF (67.79%) and followed by 10% gFF (66.66%), 5% gFF (51.67%) and 0% gFF (43.33%). In this study, significant differences ($p<0.01$) were found in the oocytes classified as M-II stages between follicular fluid supplemented 5% and control and between 5% and 10% level of follicular fluid supplementation but no significant difference ($p>0.05$) found between 10% and 15% level of follicular fluid supplementation. All the gFF supplementation groups found significantly higher ($p<0.01$) than that of the control group (0% gFF). Though the increased supplementation of gFF gradually increased the M-II, GV, GVBD and M-I are not the indicators of oocyte maturation. Similar level of GVBD was recorded in 0% to different level of gFF (Table I).

Table I. *In vitro* nuclear maturation of COCs cultured in different levels of goat follicular fluid (gFF)

Level of goat Follicular Fluid (gFF)	Total number of Normal COCs	Rate of Nuclear Maturation (%) (Mean ± SE)			
		GV	GVBD	M I	M II
Without supplementation (Control)	60	$18.28^a \pm 0.96$ (11)	$15.09^a \pm 0.87$ (9)	$23.31^a \pm 0.85$ (14)	$43.33^c \pm 3.33$ (26)
5%	62	$12.81^a \pm 1.16$ (8)	$11.29^a \pm 1.54$ (7)	$24.04^a \pm 1.79$ (15)	$51.67^b \pm 0.83$ (32)
10%	60	$6.61^b \pm 1.47$ (4)	$9.78^b \pm 2.52$ (6)	$16.66^b \pm 1.37$ (10)	$66.66^a \pm 0.00$ (40)
15%	62	$6.42^b \pm 1.54$ (4)	$9.60^b \pm 2.67$ (6)	$16.10^b \pm 1.48$ (10)	$67.79^a \pm 1.92$ (42)

a, b and c: Means with different superscripts within the same column differ significantly ($p<0.05$)
Figure in the parenthesis indicates the number of COCs observed after maturation
MI= Oocytes with nuclear materials, MII= Oocytes with nuclear materials and 2 polar body,
GVBD= Germinal vesicle break down and GV= Germinal vesicle

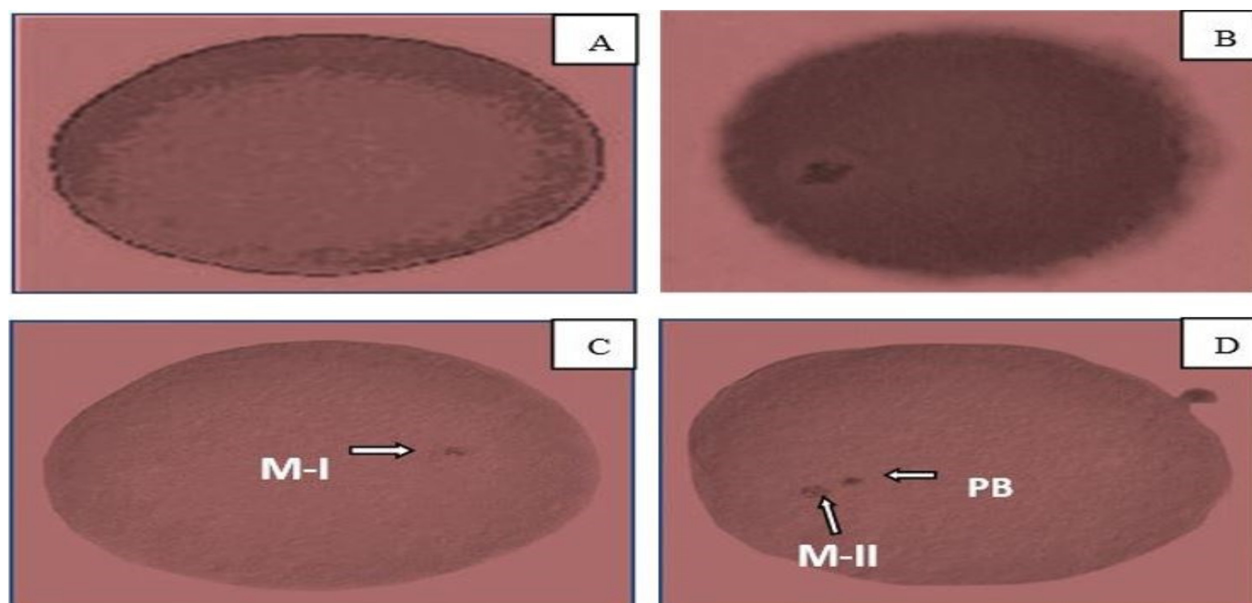

Fig. 1. Representative photograph showing different stages of maturation. A = COC with Germinal vesicle (GV), B = COC with broken germinal vesicle, C = COC in Metaphase-I (M-I) stage with nuclear materials indicated by the arrow, and D = COC in Metaphase-II stage with nuclear materials and the polar body. M-II indicated by the arrow and polar body indicated by PB.

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In Vitro Fertilization of Goat Oocyte with Fresh Semen

After maturation of COCs in TCM-199 supplemented with different levels of gFF, these COCs were fertilized with fresh buck semen. The rates of pronuclei formation is presented in Plate 2 and the result summarized in Table II. Significantly higher ($p<0.01$) percentage of normal fertilization (formation of 2 pronuclei) was observed in case of 10 and 15% gFF supplementation as 36.54 and 38.69% than control as 27.47% and 5% level as 31.69% as indicated in Table II. In this study, it was also observed that the normal fertilization rate significantly ($p<0.01$) increased and abnormal fertilization (formation of 1 and more than 2 pronuclei as well as no fertilization) significantly ($p<0.05$) decreased with gFF supplementation of 5% level compared to control. The similar significant ($p<0.01$) trend was observed in increasing level of gFF fluid to 10%, but no such significant ($p>0.05$) trend was found between 10% and 15% gFF supplementation (Table II).

Table II. *In vitro* fertilization of goat oocytes matured in media supplemented with different levels of goat follicular fluid (gFF)

Level of goat Follicular Fluid (gFF)	Total number Of Normal COCs	Fertilization rate (%) based on pronuclei (Mean ± SE)			
		NPN	1PN	2PN	PPN
Without supplementation (Control)	**80**	$67.57^{a} \pm 2.76$ (54)	$5.03^{a} \pm 1.33$ (4)	$27.47^{c} \pm 0.55$ (22)	0.00 ± 0.00 (0)
5%	**82**	$60.93^{b} \pm 4.59$ (50)	$4.89^{a} \pm 1.25$ (4)	$31.69^{b} \pm 1.09$ (26)	2.46 ± 1.23 (2)
10%	**82**	$56.78^{c} \pm 2.46$ (46)	0.00 ± 0.0 (0)	$36.54^{a} \pm 1.73$ (30)	7.31 ± 2.14 (6)
15%	**80**	$58.73^{c} \pm 0.52$ (47)	2.51 ± 1.25 (2)	$38.69^{a} \pm 2.95$ (31)	0.00 ± 0.00 (0)

a ,b and c: Means with different superscripts within the same column differ significantly (p<0.05)
Figure in the parenthesis indicates the total number
PN=Pronuclei, PPN=Polypronuclei and NPN= No pronuclei

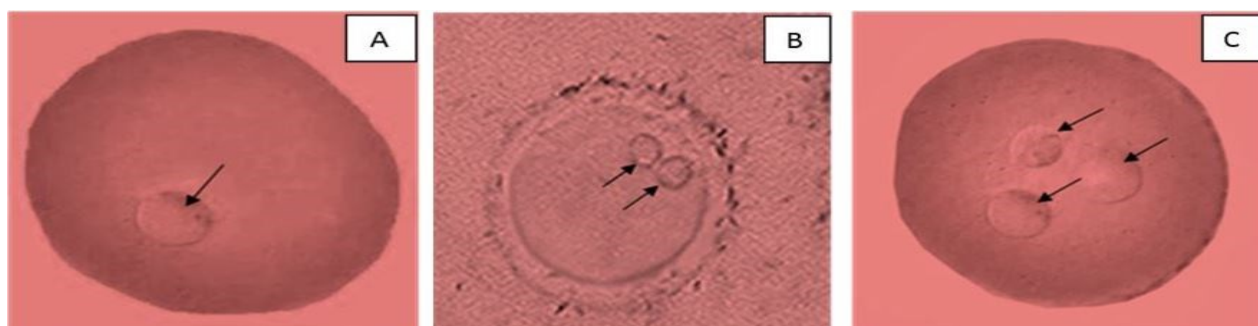

Fig. 2. Representative photograph showing fertilization. A: Oocyte with one pronucleus (1 PN), B: Oocyte with two pronuclei (2 PN) - normal fertilization, and C: Oocyte with three pronuclei (PPN) - polyspermia. Arrows indicates pronuclei.

Goat Embryos Development after 7 Days of in Vitro Culture

After fertilization, the rest of the fertilized oocytes were transferred to the culture medium TCM-199 supplemented with different level of gFF for subsequent development. The development was checked every 48 hours, and the culture was continued for 6 to 7 days. The number of 2-cell, 8-cell and compact morula from fresh semen were recorded which have been summarized in Table III and in fig. 3. Significantly higher ($p<0.01$) percentage of 2-cell, 8-cell and morula was observed in case of 5% gFF supplementation as 35.52, 23.77 and 10.17% and at 10% level of gFF as 38.31, 26.70 and 13.34% than controlled as 27.38, 19.25 and 7.95%, respectively. The percentage of 2- cell, 8-cell and morula was observed in case of 15% gFF supplementation as 37.99, 25.35 and 12.63% without having significant difference than at 10% level (Table III).

Table III. Effect of different levels of goat follicular fluid (gFF) on *in vitro* culture of goat embryos

Level of goat Follicular Fluid (gFF)	Number of Zygote	Development of embryos (%) at 7 days (Mean ± SE)			
		2-cell	8-cell	Morula	Not developed
Without supplementation (Control)	**62**	$27.38^{c} \pm 0.65$ (17)	$19.25^{c} \pm 2.37$ (12)	$7.95^{c} \pm 1.35$ (5)	$40.36^{c} \pm 1.18$ (25)
5%	**59**	$35.52^{b} \pm 2.44$ (21)	$23.77^{b} \pm 1.92$ (14)	$10.17^{b} \pm 0.17$ (6)	$23.77^{b} \pm 1.92$ (14)
10%	**60**	$38.31^{a} \pm 0.91$ (23)	$26.70^{a} \pm 1.80$ (16)	$13.34^{a} \pm 3.33$ (8)	$13.26^{a} \pm 1.38$ (8)
15%	**63**	$37.99^{a} \pm 1.70$ (24)	$25.35^{a} \pm 1.01$ (16)	$12.63^{a} \pm 1.33$ (8)	$15.97^{a} \pm 2.02$ (10)

a ,b and c: Means with different superscripts within the column differ significantly (p<0.05)
Figure in the parenthesis indicates the total number.

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Fig. 3. Representative photograph showing different stages of embryos development. A = 2-cell embryos, B = 8-cell embryos, and C = Compact morula.

IV. Discussion

In this experiment, it was observed that maturation and fertilization of COCs with gFF supplementation medium support high rates of goat embryo development by providing a beneficial effect on the goat embryo development from 2-cell to morula. The presence of gFF at 5, 10 and 15% level had positive effect on maturation, fertilization and subsequent embryo development. The percentages of COCs reached to the Metaphase-II stages were 43.33, 51.67, 66.66 and 67.79%; in case of fresh semen normal fertilization (formation of 2 pronuclei) were 27.47, 31.69, 36.54 and 38.69% for without (control), 5%, 10% and 15% level of gFF, respectively. The development of morula stages in 10% as 13.34 and 15% gFF media as 12.63. This result found significant differences ($p<0.01$) between control and 5% gFF supplemented oocytes reaching M-II stages and between 5% and 10% gFF supplemented oocytes maturation, but no significant difference ($p>0.05$) were found between 10% and 15 % levels of gFF. The beneficial effect of gFF on goat oocyte maturation may be due to the presence of growth factors, hormones and intra-ovarian peptides in more physiological properties in FF [5]. The differences among the treatments recorded in the present study between the gFF supplemented at 5% level and non-supplemented medium (control) may be due to this beneficial effect. To create an optimum environment for maximum output in IVM of oocytes, a certain level of FF is needed to be maintained in the media, above which no further improvement would occur. This trend was evident in the present study, where similar results were found between 10% and 15% gFF supplementation. Nandi [15] reported that fluid from normal follicle contains a balanced proportion of estradiol/progesterone (EPR) and insulin-like growth factor binding proteins (IGFBPs) which enhance the maturation and fertilization of oocytes. The fertilization rate is directly dependent on the maturation of oocytes. Comparing the effects of FF and oestrus cow serum, Larocca [13] reported that the presence of FF in culture medium during IVM-IVF increased the fertilization rate and percentage of morula/blastocysts. Kim [10] also observed the beneficial effects of maturation and development ability of bovine oocytes by addition of follicular fluid to the maturation medium.

V. Conclusion

The results of this experiment indicate that goat follicular fluid (gFF) at 10% level could be used as a supplement in TCM-199 maturation and culture media. It has beneficial effects on *in vitro* production of goat embryos and the rate of development of morula should be increased when 10% gFF supplement in maturation and culture media.

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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 Biotechonolo--gy, 2015. 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 ; Mobile No: +8801716902084

Dr. M. A. M Yahia Khondoker
Professor, Department of Animal breeding and Genetics, Bangladesh Agricultural University, Mymensingh-2202. Former professor, Faculty of sustainable Agriculture, University of Malaysia Sabah. Email: yahiakhabg@yahoo.com

S.A. Masudul Hoque
Assistant Professor at Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh. PhD Fellow at Hiroshima University, MSc in Animal breeding and Genetics, BSc (Hons.) in Animal Husbandry, Bangladesh Agricultural University. Email:

Sumitra Saha
Assistant professor, Department of Biotechnology, Bangladesh Agricultural University, Mymensingh-2202. MSc in Biotechnology, Wageningen university, the Netherlands. MSc in Animal breeding and Genetics, BSc (Hons.) in Animal Husbandry, Bangladesh Agricultural University. Mymensingh-2202.
Email: sumitra.biotech@gmail.com

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