

## Effect of Goat Follicular Fluid on *in vitro* Production of Embryos in Black Bengal Goats

### Research Article

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### ABSTRACT

The study was undertaken to elucidate the beneficial effects of goat follicular fluid (gFF) added to maturation and culture media on *in vitro* maturation, fertilization and post-fertilization development of oocytes in Black Bengal goats. Follicular fluid and oocytes were collected from slaughter house goat's ovaries using the aspiration technique. Media were prepared using TCM-199 supplemented with 0.5% bovine serum albumin (BSA) plus four levels of gFF at concentrations of 0%, 5%, 10% and 15%. Oocytes were matured for 27 h, fertilized with capacitated fresh semen in Brackett and Oliphant (BO) medium for 6 h and then cultured up to 7 days, at 38.5 °C with 5% CO<sub>2</sub> under humidified air. It was observed with 0% to 15% of gFF that 53.8-75.0% of the oocytes reached the cumulus cell expansion level-3; 41.5-67.8% reached metaphase-II; 28.6-38.4% exhibited normal fertilization (formation of 2-pronuclei); 12.3-33.7% were 2-cell embryos. The development of embryos was arrested at the 2-cell stage in control media and at the 8-cell stage in 5% media, though morula and blastocyst stages developed in 10% (14.1% and 9.3%, respectively) and 15% gFF media (18.0% and 10.0%, respectively). In comparison, it was observed that the results in all stages of *in vitro* production of embryos could be significantly increased ( $P < 0.05$ ) by adding 5% gFF to control media. The results could further be improved ( $P < 0.05$ ) by increasing the level of gFF to 10% but no further increment ( $P > 0.05$ ) occurred when gFF increased to 15% level. It is concluded that gFF has a positive effect on *in vitro* production of embryos in Black Bengal goats and a 10% level of gFF is recommended based on the improvements observed and the associated economic benefits.

**KEY WORDS** Black Bengal goat, goat follicular fluid, *in vitro* culture, *in vitro* fertilization, *in vitro* maturation.

### INTRODUCTION

In the *in vitro* production (IVP) of mammalian embryos, the efficiency of fertilization and post-fertilization development is influenced markedly by *in vitro* maturation (IVM) conditions (Fukui and Ono, 1989). Mammalian oocytes can

spontaneously undergo germinal vesicle breakdown and subsequent formation of metaphase-II stage during maturation and almost similar development occurs in both *in vivo* and *in vitro* (Edwards, 1965). However, fertilization and subsequent development of oocytes to the pre-implantation stages following (IVM) are generally less successful than

those of oocytes matured *in vivo* (Leibfried Rutledge *et al.* 1987).

Follicular fluid (FF) is an environmental factor in which oocytes are nourished and undergo maturation *in vivo*. FF contains a variety of peptide growth factors (Knight *et al.* 1996), some of which have been suggested to play a key role in the ability of oocytes to undergo nuclear and cytoplasmic maturation (Driancourt and Thuel, 1998). Follicular fluid is reported to consist of various growth factors, follicle-stimulating hormone (FSH), leutinizing hormone and several other nutrients although it may also contains oocyte maturation inhibitory factor (Avery *et al.* 2003). The specific role of FF is unknown. However, it is reported to protect the oocyte from the factors that induce premature resumption of meiosis, guard the oocyte from proteolytic attack and facilitate its extrusion during ovulation and enhance spermatozoa attraction, motility and acrosome reaction (Avery *et al.* 2003). Follicular fluid is being successfully incorporated in IVM media of cattle (Alia *et al.* 2004), human (Chi *et al.* 1998), sheep (Shabankareh and Sarsaifi, 2008) and pigs (Ito *et al.* 2008), buffalo (Nandi *et al.* 2004), equines (Bogh *et al.* 2002) and goats (Cognié *et al.* 2004). Tropical goats are generally classified as non-seasonal with major estrous activity being concentrated during certain periods of the year (Goel and Agrawal, 2000). There is, however, not much information regarding the effect of FF on *in vitro* maturation of oocytes in tropical goats. In Bangladesh, few studies have been conducted on IVP of goat embryos where oocytes were collected by aspiration of 2-6 mm diameter follicles from slaughter house goat's ovaries, (IVM) and (IVC) were carried out in TCM-199 supplemented with 5% fetal calf serum (Ferdous, 2006; Islam *et al.* 2007; Mondal *et al.* 2008). No work has been performed using follicular fluid on (IVM) of goat's embryos. Keeping the aforesaid reality in mind the present research was undertaken to study the effects of follicular fluid supplementation in maturation media on the *in vitro* maturation, fertilization and post-fertilization development of oocytes in Black Bengal goats.

## MATERIALS AND METHODS

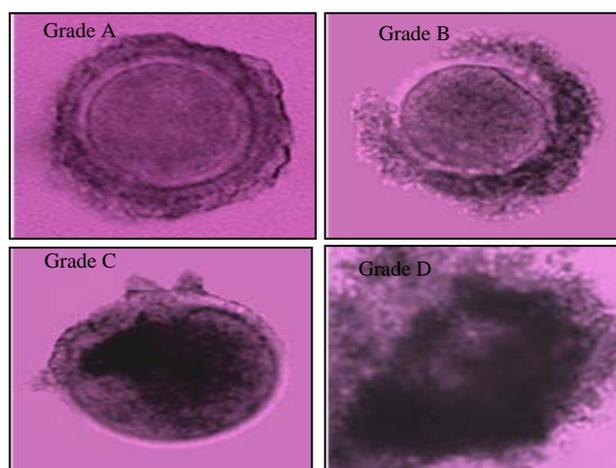
### Collection and evaluation of ovaries and oocytes

Goat ovaries were collected from a local slaughter house and transported to the laboratory in 0.9% normal saline kept in a thermo flask within 1-2 h of slaughter. 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 5 mL plastic syringe.

The aspiration medium consisted of tissue culture medium (TCM)-199 and phosphate buffered saline (PBS) sup-

plemented with 0.3% bovine serum albumin (BSA) at 1:1 ratio.

The collected COCs were classified into 4 grades on the basis of cumulus cells and nucleus 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 (Figure 1). Grade A and B oocytes were considered as normal while grade C and D as abnormal COCs.



**Figure 1** Representative photographs showing different grades of cumulus oocyte complex (COCs) (at 10X magnification of Phase contrast microscope)

Grade A: Oocytes completely surrounded by cumulus cells

Grade B: Oocytes partially surrounded by cumulus cells

Grade C: Oocytes not surrounded by cumulus cells

Grade D: Degeneration observed both in oocytes and cumulus cells

### Follicular fluid collection and preparation

Follicular fluid was collected from all categories of morphologically healthy (non atretic) surface follicles by aspiration using 10 mL syringe with 18.5 G needle. Criteria for assessment of follicular health established by Kruij and Dieleman (1982) for bovine ovaries were applied in this experiment to assess the 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 was pooled, centrifuged twice at 3000 rpm for 30 min. The supernatant was collected and filtered through a 22 µm filter (Millipore1 Corporation, Saint Quentin, France) and then transferred into a sterile glass beaker for heat inactivation at 56 °C for 1 hour in a water bath. Finally the fluids were stored in sterile 1.5 mL. capacity micro-centrifuge tubes at -20 °C for subsequent use for (IVM).

### ***In vitro* maturation (IVM) of COCs**

Media were prepared using TCM-199 (Sigma Chemicals Co., St Louis, MO, USA) supplemented with 0.5% BSA (Sigma Chemicals Co., St Louis, MO, USA), 1% penicillin plus gFF in concentration of 0%, 5%, 10% and 15% level separately. Normal quality COCs (A and B grade) were washed 2-3 times with the aspiration medium and twice in the TCM-199 medium supplemented with 2.5% BSA. Then COCs were randomly divided into four groups. Group 1 (n=160) COCs were cultured in 0% gFF, Group 2 (n=164) were in 5% gFF, Group 3 (n=162) were in 10% gFF and Group 4 (n=164) were in 15% gFF media. About 10 to 12 oocytes from each group were transferred into 50  $\mu$ L droplets of respective maturation media in 30 mm culture dish. The droplets containing oocytes were covered with warm (38.5 °C) mineral oil (Labo America, Inc., California, USA) and then the culture dish were placed in a CO<sub>2</sub> incubator (38.5 °C, 5% CO<sub>2</sub> in air, 90-95% relative humidity) and cultured for 27 h.

#### **a) Macroscopic observation of cumulus cell expansion**

After 27 h of IVM, macroscopic maturation was determined by the examination of three levels of cumulus expansion under phase contrast microscope at 10x magnification as developed by [Rahman \*et al.\* \(2004\)](#) for bovine oocytes. Briefly; level-1: indicating less expansion of COCs; level-2: indicating moderate expansion and level-3: indicating marked expansion of cumulus cells with a compact layer or chorona radiata.

#### **b) Nuclear maturation**

After observing cumulus cell expansion, 25% of the cultured COCs from each droplet was taken randomly 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 and examined under phase contrast microscope at high magnification (100x) with emersion oil for germinal vesicle break down (GVBD), metaphase-I (M-I) and metaphase-II (M-II) stage.

### ***In vitro* fertilization (IVF)**

The semen was collected by artificial vagina (AV) method from the bucks of USDA funded BBG project, Department of Animal Breeding and Genetics, BAU, Bangladesh and washed in Brackett and Oliphant (BO) medium containing 10  $\mu$ g/mL heparin and then centrifuged twice at 500 rpm for 5 min. The sperm cells were suspended in BO medium containing 0.5% BSA, 1% penicillin, 10  $\mu$ g/mL heparin and 2.5 mM caffeine and the sperm concentration was adjusted to  $1 \times 10^6$  per mL. Then the processed semen placed in a culture dish and 100  $\mu$ L droplets prepared, covered with

mineral oil and placed in a CO<sub>2</sub> incubator for 5 h at 38.5 °C for capacitation. After that, the COCs were transferred to each of the sperm drops prepared and cultured for 6 h in the same incubator. After 5 hours of incubation, 25% of total COCs initially used for maturation were randomly taken from each drop and 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 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.

### **Statistical analysis**

Simple analysis of variance (ANOVA) in completely randomized design (CRD) was performed and for comparing means, Duncan's multiple range test (DMRT) was applied by means of the Statistical Analysis System ([SAS, 1998](#)).

## **RESULTS AND DISCUSSION**

### ***In vitro* maturation (IVM) of COCs**

#### **a) Cumulus cell expansion of COCs after 27 h culture**

The results of the cumulus cell expansion of COCs cultured in TCM-199 supplemented with different levels of goat follicular fluid are presented in Table 1 and Figure 2. The results showed that the rate of cumulus cell expansion in level-3 could be significantly ( $P < 0.01$ ) increased from 51.5% to 60.3% (Table 1) by supplementing 5% level of follicular fluid (FF) to control media. The expansion rate could further be significantly ( $P < 0.01$ ) increased to 73.9% by increasing the FF supplementation up to 10% but no significant improvement (75.0%) was observed when the level of follicular fluid increased to 15% (Table 1).

#### **b) *In vitro* nuclear maturation**

The result of nuclear maturation of COCs cultured in different levels of goat follicular fluid (FF) is presented in Table 2 and Figure 3. 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 but no significant difference ( $P > 0.05$ ) found between the follicular fluid level of 10% and 15% (Table 2).

### ***In vitro* fertilization (IVF)**

The results of pronuclei formation at 6 h of fertilization are summarized in Table 3 and Figure 3.

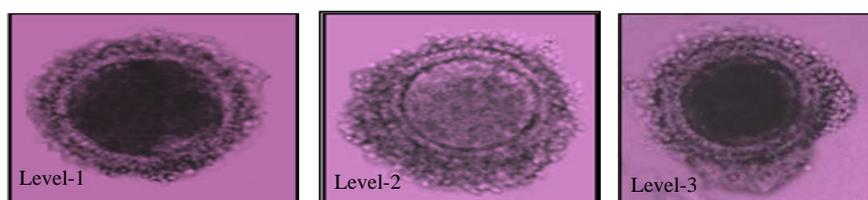
**Table 1** Cumulus cell expansion of COCs cultured in maturation media supplemented with different levels of goat follicular fluid

Level of follicular fluid	Total number of oocytes	Expansion level		
		(Level-1)	(Level-2)	(Level-3)
0% (Control)	160	23.7 <sup>a</sup> ±1.58 (38)	24.8 <sup>a</sup> ±1.30 (40)	51.5 <sup>c</sup> ±0.60 (82)
5%	164	16.9 <sup>b</sup> ±1.52 (28)	22.8 <sup>a</sup> ±1.34 (38)	60.3 <sup>b</sup> ±0.45 (98)
10%	162	7.7±1.72 <sup>c</sup> (12)	19.4 <sup>b</sup> ±1.26 (30)	73.9 <sup>a</sup> ±0.64 (120)
15%	164	8.7 <sup>c</sup> ±1.57 (14)	17.3 <sup>b</sup> ±1.29 (28)	75.0 <sup>a</sup> ±0.51 (122)

Values are shown in Mean±SE.

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Figure in the parenthesis indicates the total number of (COCs).



**Figure 2** Representative photographs showing different levels of expansion of cumulus cells after 27 h of maturation (at 10X magnification of Phase contrast microscope)

Level-1: Indicating less expansion of COCs; Level-2: Indicating moderate expansion and Level-3: Indicating marked expansion of cumulus cells with a compact layer

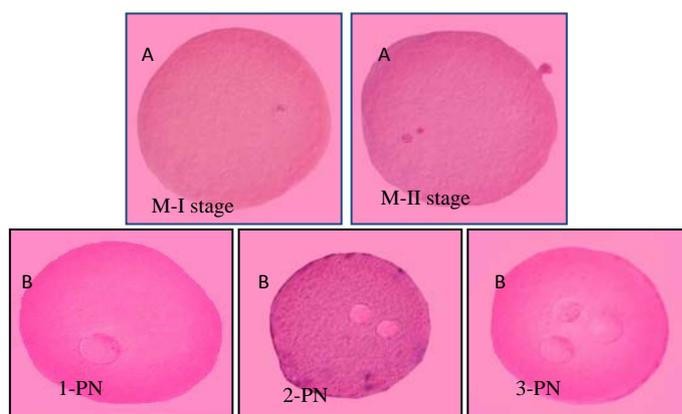
**Table 2** *In vitro* nuclear maturation of COCs cultured in maturation media supplemented with different levels of goat follicular fluid

Level of follicular fluid	Total number of oocytes	Nuclear maturation rare (%)			
		M-I	M-II	GVBD	Others
0% (Control)	40	22.6 <sup>a</sup> ±0.91 (9)	44.8 <sup>c</sup> ±1.01 (18)	14.8 <sup>a</sup> ±0.87 (6)	17.8 <sup>a</sup> ±1.37 (7)
5%	41	22.8 <sup>a</sup> ±0.87 (9)	53.8 <sup>b</sup> ±0.89 (22)	12.3 <sup>a</sup> ±0.68 (5)	12.1 <sup>a</sup> ±1.31 (5)
10%	40	17.3 <sup>b</sup> ±0.76 (7)	66.8 <sup>a</sup> ±0.87 (26)	9.9 <sup>b</sup> ±0.74 (4)	5.00 (2)
15%	41	17.9 <sup>b</sup> ±0.85 (7)	67.8 <sup>a</sup> ±0.99 (28)	10.4 <sup>b</sup> ±0.87 (4)	4.9 (2)

Values are shown in Mean±SE.

The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Figure in the parenthesis indicates the total number of oocytes.



**Figure 3** Representative photographs showing different stages of nuclear maturation after 27 h of maturation (A) and different stages of pronucleus development (B) after 6 h of fertilization (both at 100X magnification of Phase contrast microscope)

M-I: Oocytes with nuclear materials; M-II: Oocytes with nuclear materials and 2 polar body

Oocyte with one PN-asynchronous PN development / parthenogenetic activation or one PN was obscured by lipid droplets; Oocyte with two PN-normal fertilization and Oocyte with more than two PN-polyspermia

It was observed that significantly higher ( $P < 0.01$ ) percentage of normal fertilization (formation of 2 pronuclei) occurred in the oocytes matured in TCM-199 media supplemented with 10 and 15% gFF (37.60 and 38.40%, respectively) followed by 5% (32.49%) and non-supplemented media (28.61%) as indicated in Table 3.

**In vitro culture (IVC)**

The results of the development of embryos from 2-cell to morula and blastocysts up to 7 days of culture are summarized in Table 4 and Figure 4. It was observed that the development of embryos was arrested at 2-cell stage in control media and at 8-cell stage in 5% media, though morula

**Table 3** *In vitro* fertilization of oocytes matured in media supplemented with different levels of goat follicular fluid

Level of follicular fluid	Total number of oocytes	Fertilization rate (%) based on pronuclei (PN)			
		1 PN	2 PN	>2 PN	No PN
0%	40	5.0 (2)	28.6 <sup>c</sup> ±0.91 (11)	0.0 (0)	68.4 <sup>a</sup> ±0.77 (27)
5%	41	4.9 (2)	32.5 <sup>b</sup> ±0.71 (13)	2.4 (1)	60.2 <sup>b</sup> ±0.95 (50)
10%	40	0.0 (0)	37.6 <sup>a</sup> ±0.82 (30)	7.3.0±0.09 (3)	56.1 <sup>c</sup> ±0.80 (23)
15%	41	2.4 (1)	38.4 <sup>a</sup> ±0.81 (16)	0.0 (0)	59.2 <sup>c</sup> ±0.75 (24)

Values are shown in Mean±SE.

The means within the same column with at least one common letter, do not have significant difference ( $P > 0.05$ ).

Figure in the parenthesis indicates the total number of oocytes.

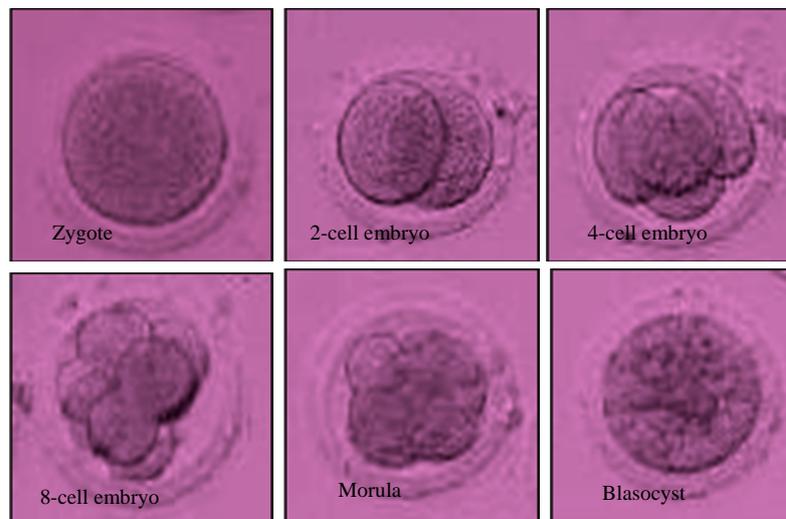
**Table 4** *In vitro* culture of oocytes matured in media supplemented with different levels of goat follicular fluid

Treatment (Level of gFF)	Number of zygote	Development of embryos (%)			
		2 cell	8 cell	morula	Blastocyst
0%	80	24.3 <sup>c</sup> ±1.23 (19)	0.0 (0)	0.0 (0)	0.0 (0)
5%	81	31.2 <sup>b</sup> ±0.95 (25)	4.3 <sup>a</sup> ±1.96 (3)	0.0 (0)	0.0 (0)
10%	82	33.2 <sup>a</sup> ±0.88 (27)	21.2 <sup>a</sup> ±0.91 (17)	14.1±0.75 (11)	9.3±0.93 (7)
15%	80	33.7 <sup>a</sup> ±1.26 (27)	22.0 <sup>b</sup> ±2.69 (18)	18.0±0.71 (14)	10.0±1.21 (8)

Values are shown in Mean±SE.

The means within the same column with at least one common letter, do not have significant difference ( $P > 0.05$ ).

Figure in the parenthesis indicates the total number of oocytes.



**Figure 4** Representative photographs showing different stages of embryonic development from zygote to blastocyst up to 7 days of culture (at 40X magnification of Phase contrast microscope using emersion oil)

and blastocyst stages were developed in 10% (14.11% and 9.31%, respectively) and 15% gFF media (18.01% and 10.03%, respectively).

*In vitro* production (IVP) of embryos refers to the use of laboratory systems for the production of embryos that will later be used *in vivo*. This process usually included collection of oocytes from the ovarian follicles of a female, *in vitro* maturation (IVM), fertilization (IVF) of oocytes and *in vitro* culture (IVC) of presumptive zygotes to the morula or blastocyst stage (Brackett *et al.* 1982). Although goat oocytes can be recovered in relatively large numbers from abattoir ovaries, the oocytes frequently have reduced development potential compared to *in vivo* matured or immature oocytes collected after gonadotropin treatment (Cognié *et al.* 2003). Poor development potential starts with maturation that limits the suitability of these oocytes for biotechnology application and slows the application of *in vitro* embryo production to commercial scale for the purpose of embryo transfer (Khandoker *et al.* 2001). 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 (Rahman *et al.* 2008). Maturation media and supplementation plays a vital role on developmental potential of oocytes in IVP procedure. Generally buffered Tissue Culture Medium-199 (TCM-199) is used as a basic medium for IVM of goat oocytes but to establish well-defined medium scientists added hormones, vitamins along with different protein supplements. Protein supplements are important for optimum maturation and subsequent fertilization. A lot of experiments have been conducted on different protein supplements, for example, fetal bovine serum (FBS) (Tajik and Esfandabadi, 2003), fetal calf serum (FCS) (Rho *et al.* 2001), steer serum (SS) (Jiménez-Macedo *et al.* 2007), estrus goat serum (EGS) (Kharche *et al.* 2006), estrus sheep serum (ESS) (Kharche *et al.* 2009), bovine serum albumin (BSA) (Rajikin *et al.* 1994), peritoneal fluids from rabbit or goat (Malik *et al.* 1999), follicular cells (Jiménez-Macedo *et al.* 2005) and follicular fluids (Cognié *et al.* 2004).

In the present research goat follicular fluid (gFF) was used in maturation media TCM-199 to find out the suitability of gFF as additional supplement. It was observed that the rates of cumulus cell expansion to level-3 and *in vitro* maturation (IVM) were significantly ( $P < 0.01$ ) increased when gFF was added at 5% level. The rates were further increased significantly when gFF was increased to 10% but no significant improvement was observed when the level of follicular fluid was increased to 15%. Supplementation of FF from non-atretic or gonadotrophin-stimulated large follicles ( $>4\text{mm}$ ) had some beneficial effect in goat oocytes (Martino *et al.* 1995; Cognié *et al.* 2004). This beneficial effect on goat oocyte maturation may be due to the pres-

ence of growth factors, hormones and intra-ovarian peptides in more physiological proportions in FF (Cognié *et al.* 2004). The differences among the treatments recorded in the present study between the FF supplemented at 5% level and non-supplemented medium (control) may be due to this beneficial effect.

To create optimum environment for maximum output in IVM of oocytes, a certain level of FF needed to be maintained in the media, above which no further improvement would occur. This theory was evident in the present study, where similar results were found between 10% and 15% FF supplementation. Maturation rate using 10-15% level of gFF averaging 67% was almost similar to the maturation rate of oocytes in TCM-199 supplemented with 10% fetal bovine serum (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). *In vitro* fertilization showed similar results whereby higher fertilization rates were observed when oocytes were cultured in media supplemented with 10% and 15% level of gFF followed by 5% and 0% level. The difference between 10% and 15% level of gFF was insignificant indicating that the effects of additional gFF has reached a plateau. The results of the present study WERE comparable to the observation by Mondal *et al.* (2008) who reported 27.8-38.2% fertilization rates in goat IVF when 5% fetal calf serum supplementation was added to maturation media. Rodriguez *et al.* (2001) reported that the percentage of goat oocytes with two pronuclei in IVF was 21.0-39.7% which was consistent with the result presented here. In pigs, FF induced oocyte maturation *in vitro* and improved the rate of male and female pronuclei formation and subsequent developmental capacity (Naito *et al.* 1989; Yoshida *et al.* 1992).

Comparing the effects of FF and oestrous cow serum, Larocca *et al.* (1993) reported that the presence of FF in culture medium during IVM-IVF of bovine oocytes increased the fertilization rate and percentage of morulae / blastocysts. Kim *et al.* (1996) also observed the beneficial effects of the addition of follicular fluid to the maturation medium on the maturation and developmental ability of bovine oocytes.

In humans, the addition of mature FF to IVM medium of immature oocytes from unstimulated ovaries was shown to increase both the fertilization rates and embryo quality (Cha *et al.* 1991). High concentrations of steroid hormones in the follicular fluid have a positive correlation with nuclear and cytoplasmic maturation of oocytes in stimulated women (Lobo *et al.* 1985) and in the rhesus monkey (Morgan *et al.* 1990). In humans, intrafollicular FSH was suggested by Suchanek *et al.* (1994). To act synergistically with oestradiol to enhance cytoplasmic maturation, resulting in successful fertilization In the present study, the development of

embryos was arrested at 2-cell stage when cultured without gFF, but a few developed to 8-cell stage when cultured in a media containing 5% gFF.

This problem of arrested development could be resolved by increasing the level of gFF supplementation to 10%, where it was observed that embryos were able to develop to morula and blastocyst stage. Increasing gFF to 15% resulted in the same trend as was with oocyte maturation. The results of the *in vitro* culturing of embryos were comparable with the results of Mondal *et al.* (2008) who reported 6.9% to 25.6% morula and 3.4% to 12.8% blastocyst development in Black Bengal goat. Gardner *et al.* (1994) found 29% development rate of sheep oocytes to compact morula while John *et al.* (2000) recorded 20-32% developmental rate to morula stage in goat oocytes.

## CONCLUSION

After the above discussion, we could conclude that gFF has a positive effect on *in vitro* maturation, fertilization and post-fertilization development of Black Bengal goat oocytes. Since 10% and 15% level of gFF showed similar results, we could recommend that 10% level of gFF could be used to supplement TCM-199 media for *in vitro* production of embryos in Black Bengal goat.

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