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

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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% CO₂ 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 Sarai, 2008) and pigs (Ito et al. 2008), buffaloes (Nandi et al. 2004), equines (Boh et al. 2002) and goats (Cognie 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. 2003). 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) supplemented 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](image)

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 Kruip 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 µL 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 CO2 incubator (38.5 °C, 5% CO2 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 µg/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 µg/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 µL droplets prepared, covered with mineral oil and placed in a CO2 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– polysperma.

### 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).

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**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% \((\text{Table 1})\) 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% \((\text{Table 1})\).

#### 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% \((\text{Table 2})\).

**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

<table>
<thead>
<tr>
<th>Level of follicular fluid</th>
<th>Total number of oocytes</th>
<th>Expansion level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Level-1)</td>
</tr>
<tr>
<td>0% (Control)</td>
<td>160</td>
<td>23.7±1.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>5%</td>
<td>164</td>
<td>16.9±1.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(28)</td>
</tr>
<tr>
<td>10%</td>
<td>162</td>
<td>7.7±1.72c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12)</td>
</tr>
<tr>
<td>15%</td>
<td>164</td>
<td>8.7±1.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(14)</td>
</tr>
</tbody>
</table>

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).

![Representative photographs showing different levels of expansion of cumulus cells after 27 h of maturation](image)

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

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

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.

![Representative photographs showing different stages of nuclear maturation after 27 h of maturation](image)

M-I: Oocytes with nuclear materials; M-II: Oocytes with nuclear materials and 2 polar bodies
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-polysperma
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.

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

<table>
<thead>
<tr>
<th>Level of follicular fluid</th>
<th>Total number of oocytes</th>
<th>Fertilization rate (%) based on pronuclei (PN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 PN</td>
</tr>
<tr>
<td>0%</td>
<td>40</td>
<td>5.0</td>
</tr>
<tr>
<td>5%</td>
<td>41</td>
<td>4.9</td>
</tr>
<tr>
<td>10%</td>
<td>40</td>
<td>0.0</td>
</tr>
<tr>
<td>15%</td>
<td>41</td>
<td>2.4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment (Level of gFF)</th>
<th>Number of zygote</th>
<th>Development of embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 cell</td>
</tr>
<tr>
<td>0%</td>
<td>80</td>
<td>24.3±1.23</td>
</tr>
<tr>
<td>5%</td>
<td>81</td>
<td>31.2±0.95</td>
</tr>
<tr>
<td>10%</td>
<td>82</td>
<td>33.2±0.88</td>
</tr>
<tr>
<td>15%</td>
<td>80</td>
<td>33.7±1.26</td>
</tr>
</tbody>
</table>

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 oocytes 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 (>4mm) 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 presence 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 resolve by increasing the level 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 oocytes 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 result, 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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