Parthenogenetic Activation of Black Bengal Goat Oocytes

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ABSTRACT

In vitro maturation and activation of oocytes are primary steps towards biotechnological manipulation in embryology. The objectives of the present study were to determine the oocyte recovery rate per ovary, *in vitro* maturation rates of oocytes and rates of parthenogenetically activation of matured oocytes in Black Bengal goats. All visible follicles were aspirated to recover follicular fluid from individual ovaries (number of ovaries = 456). The immature cumulus oocyte complexes (COCs; n = 1289) were cultured in tissue culture medium (TCM)-199 supplemented with 10% (v/v) fetal bovine serum (FBS) for 27 hours at 39℃ with 5% CO_2 in humidified air. The matured oocytes (n = 248) were activated with 5 μM ionomycin for 5 minutes followed by treatment with 2 mM 6-dimethylaminopurine (6-DMAP) for 4 hours. After activation, oocytes were cultured for another 14 hours in TCM-199 supplemented with bovine serum albumin (BSA) at 39℃ with 5% CO_2 in humidified air. The pronucleus formation in activated oocytes was determined by staining with 1% orcein (whole mount technique). Matured oocytes (n = 176) without activation stimuli were used as control. The mean number of oocytes recovered per ovary was 3.5 ± 0.5. The proportion of oocytes matured *in vitro*, confirmed by the presence of first polar body, was 42.1 ± 4.7%. Parthenogenetic activation, evidenced by formation of pronucleus, occurred in 37.2 ± 15.8% of matured oocytes. No pronucleus formation was observed in control oocytes. In conclusion, a combination of ionomycin and 6-DMAP induces activation in one third of Black Bengal goats' oocytes.

(Key words : 6-DMAP, goat oocytes, ionomycin, parthenogenetic activation)

INTRODUCTION

In vitro production (IVP) and embryo transfer (ET) have not been practiced for the development of goats in Bangladesh. Researches on *in vitro* maturation (IVM), *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT) and *in vitro* culture (IVC) of Black Bengal goat embryos will greatly contribute to our basic understanding of reproductive physiology. Application of various assisted reproductive technologies (ARTs) will increase the population and aid in reproductive management of Black Bengal goats. However, very few researches on IVP in Black Bengal goats have been conducted elsewhere (Ferdous, 2006; Mondol *et al.*, 2008). Ferdous (2006) performed investigation on collection, grading, and evaluation of Black Bengal goat oocytes with respect to IVM. Mondol *et al.* (2008) performed investigation on oocytes retrieval, rates of IVM and *in vitro* development of IVF oocytes in Black Bengal goats. However, still there is no study on artificial activation of Black Bengal goat oocytes in Bangladesh, which is very important for conducting any research on SCNT in future.

The number of oocytes retrieved per animal is important to maximize the utilization of oocytes at IVP. The quality and quantity of oocytes retrieved per ovary or animal may vary depending on the reproductive status, age of animals and oocyte harvesting techniques (Vazquez *et al.*, 1993; Wani *et al.*, 1999; Rocha *et al.*, 2006). Moreover, for routine production of embryos *in vitro*, achievement of good rates of maturation of oocytes *in vitro* is essential.

At the time of *in vivo* fertilization, activation of oocytes by penetration of spermatozoa induces a massive calcium (Ca^{2+}) mobilization that results in the discharge of the cortical granules leading to zona hardening to prevent polyspermy and resumption of meiotic division of meiosis II (MII) chromosome leading to formation of female pronucleus. Knowing this fact, scientists usually activate oocytes artificially (commonly known as parthenogenetic activation) after transfer of either somatic or embryonic donor cells to produce cloned embryos. This parthenogenetic activation of oocytes can be performed either

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by ionomycin (Loi *et al.*, 1998), calcium ionophore (Liu *et al.*, 2002), ethanol (Loi *et al.*, 1998), strontium (Cuthbertson *et al.*, 1981) or electrical pulse followed by treatment with either 6-dimethylaminopurine (6-DMAP; Susko-Parrish *et al.*, 1994) or cycloheximide (Yi and Park, 2005) in different species . However, still there is no study on artificial activation of Black Bengal goat oocytes in Bangladesh which is very important for conducting any research on SCNT in future. Taken together, the present study was conducted in Black Bengal goats to determine the oocyte recovery rate per abattoir derived ovary, *in vitro* maturation rate of such oocytes and the parthenogenic activation rates by using ionomycin and 6-DMAP.

MATERIALS AND METHODS

The present study was conducted at the Field Fertility Clinic Laboratory, Department of Surgery and Obstetrics, Bangldesh Agricultural University, Mymensingh during the period from February 2009 to May 2009.

1. Chemicals and Media Used

All the chemicals, reagents, media, biologics and media constituents were purchased from Sigma-Aldrich Chemicals, St Louis, USA. Media and reagents were prepared under standard protocol following aseptic technique (Parrish *et al.*, 1988). The final media for *in vitro* maturation, activation and culture were filtered (0.22 μm pore size, Durapure® membrane filter, Ireland) and routinely equilibrated at 39℃ with 5% CO_2 in humidified air for at least 1 hour before used. Only cell culture tested chemicals were used to formulate the media.

2. Ovary Collection and Aspiration of Cumulus Oocyte Complexes (COCs)

Four hundred and fifty six ovaries of Black Bangle goats were collected at slaughter and transported to the laboratory in a thermo flask containing warm saline (35 to 37℃, 0.9% sodium chloride solution, w/v), supplemented with penicillin-streptomycin (125 μg/ml Streptopen® Renata Bangladesh Ltd., Dhaka, Bangladesh) within 2 to 3 hours of slaughter. The ovaries were rinsed three times in saline at 37℃. All visible follicles were aspirated with an 18-gauge hypodermic needle attached to a 10-ml disposable syringe (Steripack Disposable Syringe®, Opso Saline Ltd, Dhaka, Bangladesh). The follicular fluid was left for 5 minutes and the sediment was transferred to a 60 mm petridish (Greiner bio-one, Frickenhausen, Germany). The retrieved follicular aspirate was diluted with Tyroid's albumin lactate pyruvate-HEPES (TALP-HEPES) medium (washing medium; Parrish *et al.*, 1988) and searched for COCs under a sterio-microscope (Leica Microsystems, MZ6, Wetzlar, Germany). All available oocytes were selected for maturation culture. The COCs were rinsed three times in fresh TALP-HEPES and once in maturation medium before being incubated in 50 μl droplets of maturation medium.

3. *In Vitro* Maturation (IVM) of Oocytes

The basic medium used for oocyte maturation was tissue culture medium-199 (TCM-199, Earle's salts with L-glutamine and sodium bicarbonate). On the day of initiating maturation, TCM-199 was supplemented with 0.25 mM sodium pyruvate, 10%, (v/v) fetal bovine serum (FBS), 0.05 μg/ml bovine FSH (Sioux Biochemical, Sioux center, Iowa, USA), 5 μg/ml LH (Sioux Biochemical, Sioux center, Iowa, USA), 1 μg/ml oestradiol and 50 μg/ml gentamycin. Four 50 μl droplets of maturation medium were prepared in 35 mm petridish (Greiner bio-one, Frickenhausen, Germany) and covered with mineral oil. Ten to 12 COCs were placed in each droplet and the maturation culture was conducted for 27 hours at 39℃ in an incubator (Thermo Forma®, Marietta, Ohio, USA) with 5% CO_2 in humidified air.

4. Evaluation of Oocyte Maturation

After 27 hours of culture in the maturation medium, the COCs were examined under the stereomicroscope for cumulus cell expansion. Presumptive maturation was confirmed by the degree of cumulus expansion. To observe the first polar body extrusion as evidence of maturation of oocytes, the COCs were denuded (removal of cumulus cells) by vortexing for 2 minutes in minimum amount of washing media. After vortexing, the first polar body extrusion in matured oocytes was examined under inverted microscope (LEICA DM IRB®, Wetzlar, Germany) by moving the oocytes with a mouth controlled pipette.

5. *In Vitro* Activation of Oocytes

Oocytes having first polar body were activated by treatment with 5 μM ionomycin (Cat No. I 0634) in washing medium for 5 minutes. After washing three times in washing media, activated oocytes were treated with 2 mM 6-DMAP (Cat No. D 2629) in IVC medium for 4 hours at 39℃ with 5% CO_2 in humidified air. Matured oocytes (oocytes with first polar body) without any activation were used as control.

6. *In Vitro* Culture (IVC) of Activated Oocytes

TCM-199 supplemented with 10% (v/v) bovine serum albumin (BSA) was used as embryo culture medium. Eight to 10 oocytes were cultured in 25 μl droplets of medium covered with mineral oil. After treatment with 6-DMAP, the oocytes were transferred into IVC medium and incubated for another 14 hours at 39℃ with 5% CO_2 in humidified air. At the same time, matured oocytes without activation were cultured at similar condition as a control group.

7. Evaluation of Oocyte Activation

At 14 hours of IVC (18 hours of primary activation), oocytes were stained by whole mount technique. Briefly, five to 10 oocytes were fixed with acid ethanol (acetic acid : ethanol = 1 : 3) at room temperature on glass slide under cover slip attached with adhesive. The fixed oocytes were further fixed in acid ethanol solution overnight at 4℃ followed by staining with 1% (w/v) orcein in 45% acetic acid for 10 minutes. After cleaning the oocytes and slides with aceto-glycerol (20% acetic acid + 20% glycerol) and sealed with nail polish, the oocytes were examined for pronucleus formation under a phase contrast microscope (OLYMPUS®, BX51, Tokyo, Japan) at 400 ×.

8. Statistical Analysis

The data were stored in Microsoft Excel work sheet and different descriptive statistics were performed. The data on number of oocyte recovery per ovary, percentage of *in vitro* maturation and proportion of oocyte activation were expressed as mean ± SD.

RESULTS

The number of oocyte recovered per Black Bengal goat ovary (Fig. 1) collected at abattoir is shown in Table 1. When aspirated follicular fluid was examined for COCs, the mean number of oocytes (Fig. 2) recovered per ovary was 3.5 ± 0.5. The proportion of oocytes matured *in vitro* in Black Bengal goats is shown in Table 2. A total of 1,289 oocytes were cultured *in vitro* (number of replicates = 18) across the days. The oocytes maturation percentage as confirmed by the presence of first polar body (Fig. 3) in Black Bengal goats was 42.1 ± 4.7 %.

The proportion of oocytes activated by using ionomycin and 6-DMAP in Black Bengal goats is shown in Table 3. A total of 248 activated oocytes were examined for formation of pronucleus (Fig. 4) as evidence of oocytes activation. The proportion of oocytes activation by using ionomycin and 6-DMAP was 37.2 ± 15.8%. No pronucleus formation was observed in

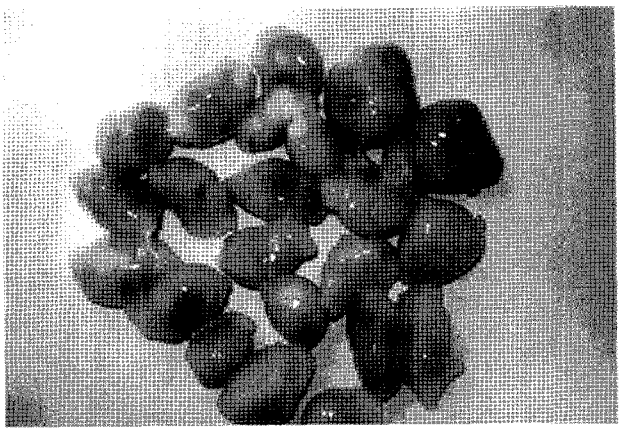

Fig. 1. Black Bengal goats' ovaries collected at slaughter.

Table 1. Number of oocyte recovered per ovary of a slaughtered Black Bengal goat

Total no. of ovaries examined	Total no. of oocytes recovered	Oocytes recovery rate per ovary (Mean ± SD)
456 (n = 18)	1,588	3.5 ± 0.5

n = Number of times sampled.

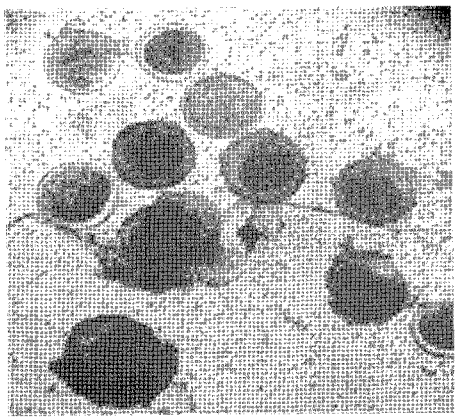

Fig. 2. Immature COCs (Inverted microscope, 40 x).

Table 2. *In vitro* maturation rates of Black Bengal goat oocytes

No. of oocytes cultured	No. of oocytes matured	Maturation rate (Mean ± SD %)
1,289 (n = 18)	539	42.1 ± 4.7

n = Number of times cultured.

Table 3. Proportion of parthenogenetically activated Black Bengal goat oocytes

Activation stimulation	No. of activated oocytes examined	No. of oocytes activated	Oocyte activation rate (Mean ± SD %)
Positive	248	87	37.2 ± 15.8
Negative (Control)	176	0	0

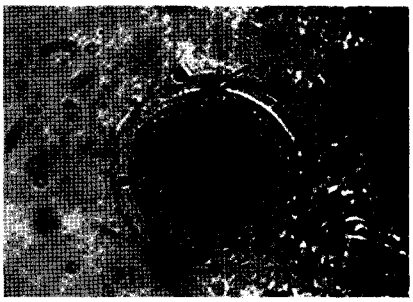

Fig. 3. An *in vitro* matured oocyte of a Black Bengal goat with first polar body (Black arrow; 200 x).

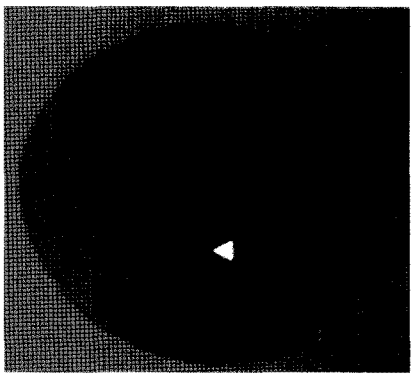

Fig. 4. Pronucleus formation in an activated oocyte of a Black Bengal goat (Black arrow indicates pronucleus and white arrow head indicates polar body; DIC microscope, 400 x).

176 matured oocytes cultured without activation stimulation.

DISCUSSION

At any IVP program, the number of oocyte recovery per ovary or animal is important to maximize the utilization of oocytes. In the present study, the rate of oocyte recovery per ovary of Black Bengal goat is 3.5 ± 0.5. The rate of oocytes recovery per ovary is higher than that recovered by previous study (Mondol *et al.*, 2008). The variation in oocyte recovery rate per ovary between two studies may be due to aspiration of follicles of particular diameter compared to aspiration of all visible follicles. Moreover, oocyte recovery rate may vary depending on the reproductive status of animals. There are reports that oocytes recovery rates per animal have not been influenced by the reproductive status as evidenced by no difference in oocyte recovery rates between pubertal and prepubertal Minke, sei and Bryde's whales (Iwayama *et al.*, 2004; Bhuiyan *et al.*, 2009). On the contrary, ovaries without corpus luteum yielded significantly higher number of oocyte than that of ovaries with corpus luteum in sheep (Wani *et al.*, 1999).

If oocytes derived from abattoir ovaries are used, satisfactory rate of oocyte maturation should be achieved in any IVP program. In the present study, maturation rate of Black Bengal Goat oocytes is 42.1 ± 4.7%. Contrasting to the present study, Mondol *et al.* (2008) found higher IVM rate than that of present study. Mondol *et al.* (2008) obtained 51.52% IVM rate in oocytes derived from ovaries with corpus luteum (CL) and 71.7% in oocytes derived from ovaries without CL. This variation in IVM rates in Black Bengal goats between two studies may be due to variations in follicular diameter used for aspiration. It has been demonstrated in goats that the rate of IVM in oocytes derived from follicles of 2 to 6 mm diameter is significantly higher (86%) than that of 1 to 1.8 mm counterpart (24%; De Smedt *et al.*, 1992). In the present study, all visible follicles were aspirated for recovery of COCs irrespective of reproductive status (CL positive vs. CL negative ovaries). Further studies are needed to optimize the conditions for IVM of Black Bengal goat oocytes to obtain satisfactory proportion of matured oocytes. Moreover, oocytes should be aspirated from follicles of 2 to 6 or 8 mm diameter.

Parthenogenetic activation or artificial activation of oocytes is very important for production of cloned embryos by somatic cell nuclear transfer or embryonic cell nuclear transfer in animals. Moreover, artificial activation is performed for satisfactory proportion of female pronuclei formation in oocytes after intracytoplasmic sperm injection (ICSI) in many species (Garcia-Rosello *et al.*, 2009). This parthenogenetic activation of oocytes can be performed either by ionomycin, calcium ionophore, ethanol or electrical pulse followed by treatment with either 6-dimethylaminopurine (6-DMAP) or cycloheximide in different species. Wang *et al.* (2002) found lower pronuclei formation (39%) in oocytes of bovine activated by ionomycin compared to activation using combined ionomycin and 6-DMAP (67%). This result suggested that activation of oocytes

by single stimulation is incomplete. Ethanol, ionomycin and calcium ionophore treatments elevate oocyte intracellular Ca^{2+} levels (Yoshida *et al.*, 1992; Deng *et al.*, 1994; Tesarik *et al.*, 1995). Pronuclei are not observed in oocytes unless they exhibit a Ca^{2+} rise during activation (Deng *et al.*, 1994; Tesarik and Sousa, 1995). Oocyte meiotic arrest at Metaphase II is sustained by high levels of maturation promoting factor (MPF) and a cytostatic factor (CSF) (Sagata *et al.*, 1989). Liu and Yang (1999) reported that meiosis restart after inactivation of MPF activity and the initial rise in Ca^{2+} seen at fertilization or at oocyte activation is responsible for inactivation of MPF activity. Repetitive elevations in intracellular Ca^{2+} are required for maintaining low levels of MPF.

In the present study, when activation of Black Bengal goat oocytes was performed by using ionomycin and 6-DMAP, 37.2 ± 15.8% oocytes were activated as evidenced by the formation of female pronucleus. Moreover, no pronucleus formation was observed in any matured oocytes cultured without activation stimulation. This indicates, activation stimulation is very important for resumption of meiotic division. In cattle, depending on the activation methods, proportion of oocyte activation varies from 29 to 52% (Suttner *et al.*, 2000). In pigs, oocyte activation measured by the presence of pronulcei varies from 22 to 74% (Ruddock *et al.*, 2000), while in rabbits activated proportion of oocyte is 10 to 38% (Liu *et al.*, 2002). Although, the proportion of activation is low in the present study, it is the first report on artificial activation of oocytes of Black Bengal goats in Bangladesh. Further studies are needed with respect to other activation agents and improved IVC conditions for obtaining satisfactory proportion of female pronuclei in oocytes of Black Bengal goats. It is concluded that 3.5 oocytes can be recovered per abattoir ovary of Black Bengal goats of which 42% is matured in vitro. A combination of ionimycin and 6-DMAP induces activation in one third of Black Bengal goats IVM oocytes.

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