



Effect of growth differentiation factor-9 and fibroblast growth factor-basic on small caprine oocyte development *in vitro*

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Received: 28 February 2018; Accepted: 6 July 2018

ABSTRACT

The study aimed to assess the effect of growth differentiation factor-9 (GDF-9) and fibroblast growth factor-basic (bFGF) on *in vitro* development of small (<126 µm diameter) caprine oocytes. Small oocytes were recovered from abattoir derived caprine ovaries and matured *in vitro* (24 h) in the presence of GDF-9 (0, 10, 20, 30 ng/ml) or bFGF (0, 10, 20, 30 ng/ml), and maturity and viability rates were assessed. A combination of both the growth factors (GDF-9, 30 ng/ml + bFGF, 20 ng/ml) was used to mature the oocytes *in vitro* (24 h). Subsequently oocytes were fertilized *in vitro* with cauda epididymis sperm processed with BO medium (2×10⁶/ml sperm). Cleavage and fertilization rates were assessed at 42 to 48 h post-insemination and morula/ blastocyst rate was assessed on 7 to 8 d post-fertilization. Maturation rate was significantly greater in oocytes cultured in media containing 30 ng/ml GDF-9 or 20 ng/ml bFGF compared to control. Maturation, fertilization, cleavage and morulae/blastocyst rates were significantly greater in oocytes cultured in combination of GDF-9 and bFGF compared to control. In conclusion, the supplementation of GDF-9 and bFGF in *in vitro* maturation (IVM) medium improved the maturation rate and embryo development of small caprine oocytes.

Key words: bFGF, Caprine, GDF-9, *In vitro* development, Oocyte

Selection and dominance of antral follicle is controlled by gonadotrophins, follicle stimulating hormone (FSH), leutinizing hormone (LH) and locally produced growth factors like activin, inhibin, and follistatin. Growth differentiation factor-9 (GDF-9) is a paracrine factor and its expression is found in almost all stages of folliculogenesis (Bayne *et al.* 2015). GDF-9 is a key regulator of normal cumulus cell function and hence, GDF-9 is perhaps a key element in the oocyte–cumulus regulatory loop. Similarly, the fibroblast growth factors (FGFs) are important for oocyte development and FGF mediated signaling regulates oocyte development competence, nuclear maturation, cumulus expansion and embryo development (Ormond *et al.* 2012, Convissar *et al.* 2017). The FGF2, also known as basic FGF (bFGF), has been assessed for its functional role during oocyte maturation. It is produced by theca and granulosa cells, and its transcript abundance increases during periovulatory follicle growth and during bovine IVM (Zhang *et al.* 2010, Mondal *et al.* 2015).

The main hurdle in production of embryos in laboratory is the maturation of immature oocytes with reference to use of suitable culture conditions. Studies to unravel the

role of different growth factors in oocyte growth/ maturation would pave way for understanding the mechanism of oocyte growth/ maturation which will facilitate the utilization of small oocytes that are available in larger numbers compared to very few larger oocytes (suitable for IVF). This would result in large scale laboratory/ commercial production of embryos. Hence, the present study was conducted to examine the effect of 2 growth factors (GDF-9 and bFGF) on *in vitro* development of small caprine oocytes.

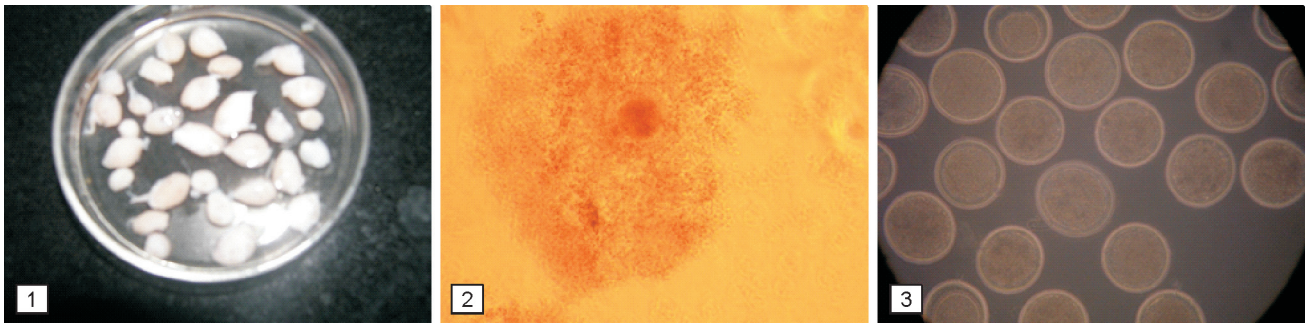
MATERIALS AND METHODS

All media and chemicals were procured from Sigma Chemicals (St Louis, MO, USA) unless otherwise stated.

Collection and processing of ovaries: Goat ovaries collected from slaughterhouse were brought to laboratory within 1 h in normal saline (0.9%) in thermo flask (32–35°C) containing 50 µg/ml gentamicin. At laboratory, the ovaries were washed in Dulbecco's Phosphate Buffered Saline (pH 7.4).

Oocytes collection and grading: Oocytes were aspirated from all surface follicles of the ovaries with a 22-G hypodermic needle attached to a syringe. The aspiration medium consisted of TCM-199 + FBS (10%) and PBS + BSA (1%) in 1: 1 ratio. Oocytes were graded by morphological appearance of the cumulus cells investments and homogeneity of ooplasm under zoom stereomicroscope, and oocytes with more than 4 layers of compact cumulus

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Figs 1–3. 1. Goat ovaries collected for the study. 2. *In vitro* matured caprine oocyte. 3. Caprine blastocysts obtained in the study.

cells and with granular homogenous ooplasm were chosen for the experiments. The diameter (including zona pellucida) was measured with the help of an embedded oculometer in stereo zoom microscope and oocytes having diameter less than 126 μm were considered as small oocytes and used in the present study (Fig. 1).

IVM of oocytes: Media for different experiments was freshly prepared and pre-incubated in a CO_2 incubator (5% CO_2 in air, humidified atmosphere) at 38.5°C for at least 2 h before use. The oocytes were washed 5–6 times in washing medium and twice with maturation medium (TCM-199 with Fetal bovine serum (10%), pFSH (0.02 IU/ml) and gentamicin (50 $\mu\text{g}/\text{ml}$)). The washed oocytes were transferred into 50 ml droplets (10–12 oocytes in a group/droplet) of maturation media. The oocytes were checked for the vitality using the trypan blue (0.05% for 2 min) staining technique. Oocytes that did not absorb the stain were considered viable (Gupta *et al.* 2002a). The maturation rates of oocytes were evaluated as per the methods described by Gupta *et al.* (2002b) (Fig. 2).

Processing of sperm: Semen was obtained from cauda epididymus of mature buck testis. Semen was centrifuged with BO medium for 6 min at 300 g and the supernatant was discarded. The process was repeated again and the pellet was dissolved in BO medium. The sperm concentration was adjusted to $2 \times 10^6/\text{ml}$ before *in vitro* fertilization with the matured oocytes and kept in a CO_2 incubator (5% CO_2 tension, humidified atmosphere) at 38.5°C for 5–10 min for swim up.

***In vitro* fertilization and embryo culture:** The medium in the maturation droplets (containing the matured oocytes) was removed and replaced by spermatozoa in the *in vitro* fertilization (IVF) medium (TCM-199 with fetal bovine serum (10%) and gentamicin (50 $\mu\text{g}/\text{ml}$)). The dishes were then placed in a CO_2 incubator (5% CO_2 in air, humidified atmosphere) at 38.5°C for 16 h. After incubation, the medium and unattached sperms were removed and replaced by TCM-199 medium supplemented with 10% FBS. Afterwards the dishes were placed again in a CO_2 incubator at 38.5°C and incubated further for 32 h. Following 40–42 h after fertilization, the presumptive zygotes were evaluated under stereozoom microscope at 110 \times magnification for the evidence of cleavage. Results were recorded in terms of cleavage rate (percentage of oocytes subjected to IVF that were cleaved to 2-cell stage).

The uncleaved oocytes/ zygotes at 24 h post-fertilization were washed with TCM-199 media supplemented with 10% FBS to remove the attached cumulus cells and spermatozoa. They were then transferred to grease-free glass slides and the extra medium was removed with the help of filter paper. Small drops of mountant (Vaseline – wax mixture) were placed on 4 corners of the cover slips. The cover slips were placed over the zygotes/oocytes. The slides were then placed in fixative for 24 h. After 24 h of fixation, oocytes/zygotes were stained with 1% aceto-orecin and were examined immediately under phase contrast microscope. Oocytes with two polar bodies in perivitelline space, with decondensed sperm nucleus and their associated tail(s), pronucleus (ei) in the ooplasm and those that cleaved after 40–42 h of insemination were together designated as fertilized. The cleaved embryos were removed from the fertilization droplets after 48 h of insemination and were further cultured at 38.5°C for 7 days in embryo culture medium (similar to IVF medium). On days 3 and 5, 40 μl of the culture medium was replaced by same quantity of fresh medium. An embryo was classified as a blastocyst (Fig. 3) upon formation of blastocoel, zona thinning and pronounced differentiation of outer trophoblast layer and inner cell mass.

Experiment 1 (To study the effect of GDF-9 and bFGF on IVM of small caprine oocytes): Viable oocytes were cultured in maturation medium in a CO_2 incubator at 38.5°C in the presence of growth factors, GDF-9 or bFGF at a concentration of 0, 10, 20 or 30 ng/ml in medium for 24 h. Oocytes in control group were cultured in maturation medium without growth factor supplementation. The viability and maturation rates were examined.

Experiment 2 (To study the effect of combined dose of GDF-9 and bFGF on cleavage rate and morulae/blastocyst yield): Based on the result of the experiment 1, single dose of GDF-9 and bFGF for IVM of goat oocytes were chosen. Oocytes were matured in maturation medium supplemented with GDF-9 (30 ng/ml) and bFGF (20 ng/ml). Oocytes in control group were cultured in maturation medium without growth factor supplementation. The procedure of IVF (as mentioned above) was same for both control and treatment groups. The maturation rates, viability, fertilization and cleavage rates were recorded. The cleaved embryos were further cultured in media containing TCM-199 + FBS (10%) + gentamicin (50 $\mu\text{g}/\text{ml}$) for 7–8 d for the evaluation of morula and blastocyst stages.

Statistical analysis: Results were expressed as means \pm SEM. A value of $P < 0.05$ was considered statistically significant. The maturation rates, viability, fertilization rates, cleavage and morulae/ blastocysts were analysed by ANOVA followed by Tukey's multiple comparison tests as *post-hoc* (the percentage values were transformed to arcsine values before analysis). The statistical software Graph Pad Prism version 6 (San Diego, USA) was used for analyzing the data.

RESULTS AND DISCUSSION

The effect of different doses of GDF-9 on IVM and viability rates of small caprine oocytes is presented in Table 1. Maturation rate was significantly ($P > 0.05$) higher in oocytes cultured in media containing 30 ng/ml of GDF-9 as compared to control. The effect of different doses of bFGF on IVM and viability rates of small caprine oocytes is presented in Table 2. Maturation rate was not different ($P > 0.05$) in oocytes cultured in control media or in media containing 10 ng/ml of bFGF, but it was significantly greater ($P > 0.05$) in oocytes cultured in media containing 20 ng/ml

Table 1. Effect of different doses of GDF-9 on *in vitro* maturation and viability rates of caprine oocytes (10 replicates).

| Treatment | Oocyte cultured (N) | Maturation rate (% \pm SEM) | Viability rate (% \pm SEM) |
|------------------|---------------------|-------------------------------|-------------------------------|
| Control | 124 | 73.98 \pm 1.43 ^a | 93.73 \pm 1.16 ^a |
| GDF-9 (10 ng/ml) | 104 | 74.90 \pm 1.97 ^a | 94.01 \pm 1.82 ^a |
| GDF-9 (20 ng/ml) | 114 | 78.72 \pm 1.99 ^a | 95.30 \pm 1.67 ^a |
| GDF-9 (30 ng/ml) | 122 | 86.26 \pm 1.53 ^b | 95.99 \pm 1.45 ^a |

Values within column without common superscripts differ significantly ($P < 0.05$).

Table 2. Effect of different doses of bFGF on *in vitro* maturation and viability rates of caprine oocytes (10 replicates)

| Treatment | Oocyte cultured (N) | Maturation rate (% \pm SEM) | Viability rate (% \pm SEM) |
|-----------------|---------------------|-------------------------------|-------------------------------|
| Control | 122 | 73.75 \pm 1.02 ^a | 94.40 \pm 1.31 ^a |
| bFGF (10 ng/ml) | 108 | 74.05 \pm 1.48 ^a | 95.43 \pm 1.69 ^a |
| bFGF (20 ng/ml) | 108 | 80.04 \pm 0.98 ^b | 96.08 \pm 1.65 ^a |
| bFGF (30 ng/ml) | 114 | 81.19 \pm 1.06 ^b | 94.86 \pm 1.53 ^a |

Values within column without common superscripts differ significantly ($P < 0.05$).

Table 3. Effect of GDF9 and bFGF in combination on viability rate, maturation rate, fertilization rate, cleavage rate and morulae/ blastocyst yield from small caprine oocytes

| Treatment | Oocyte cultured (N) | Maturation rate (% \pm SEM) | Viability rate (% \pm SEM) | Fertilization rate (% \pm SEM) | Cleavage rate (% \pm SEM) | Morulae/Blastocyst rate (% \pm SEM) |
|------------------------------------|---------------------|-------------------------------|-------------------------------|----------------------------------|-------------------------------|---------------------------------------|
| Control | 116 | 95.16 \pm 1.69 ^a | 74.20 \pm 2.46 ^a | 59.62 \pm 1.46 ^a | 41.52 \pm 2.55 ^a | 18.20 \pm 2.84 ^a |
| GDF-9 (30 ng/ml) + bFGF (20 ng/ml) | 122 | 95.36 \pm 1.70 ^a | 83.16 \pm 2.16 ^b | 67.15 \pm 2.36 ^b | 50.24 \pm 3.13 ^b | 28.18 \pm 1.45 ^b |

Values within column without common superscripts differ significantly ($P < 0.05$).

and 30 ng/ml of bFGF. No difference ($P > 0.05$) in the viability rate was observed among the control and GDF-9 (Table 1) or bFGF treated groups (Table 2). Viability rates were found approximately 95% after maturation. The effect of GDF-9 and bFGF in combination on IVM, viability and development rates of small caprine oocytes is presented in Table 3. Maturation, fertilization, cleavage and morulae/ blastocysts rates were significantly greater ($P > 0.05$) in oocytes matured in the presence of GDF-9 (30 ng/ml) and bFGF (20 ng/ml) compared to control group.

Any method like aspiration or slicing of ovaries for oocyte recovery from abattoir derived ovaries usually yield large number of small oocytes compared to its larger counterpart. Nevertheless, the small oocytes are not preferred starting materials for *in vitro* embryo production. Development potential of the small oocytes is generally compromised and only a small proportion of those are capable to develop into blastocyst following IVM, IVF and IVC (Chang *et al.* 2005). In the current study, attempts were made to improve the *in vitro* development capability of the small caprine oocyte through GDF-9 and bFGF supplementations into IVM medium. The results indicated that IVM rate of the small oocytes was significantly increased with GDF-9 or bFGF supplementation. Moreover, the rates of fertilization, cleavage and morula/blastocyst development of the small oocytes were significantly improved when IVM medium was supplemented with GDF-9 and bFGF combination.

Maturation of oocytes is controlled by several paracrine and autocrine factors that are produced within the follicular niche as well as oocyte-secreted factors (Binelli and Murphy, 2010). One of the strategies to improve the *in vitro* development competence of COC is to supplement specific paracrine and endocrine component or oocyte secreted factor into the culture media. Supplementation of GDF-9 during IVM improves the post-fertilization embryo development and post-transfer survival. GDF9 participates in cumulus expansion and stimulates MPF and MAPK activities in porcine oocytes during IVM and thus confers better development competence to the oocytes (Gilchrist *et al.* 2006, Christine *et al.* 2008). Similarly, it is believed that FGFs are important for oocyte developmental process. FGF signaling regulates oocyte development competence, nuclear maturation, cumulus expansion and embryo development (Ormond *et al.* 2012). Several FGFs are produced in oocytes and follicular somatic cells, and their receptors are present within oocytes, and granulosa and

cumulus cells (Zhang *et al.* 2010, Zhang and Ealy 2012). Although FGFs are involved in several biological processes related to folliculogenesis and proliferation and survival of granulosa and cumulus cells, specific role of various FGFs during the final period of oocyte maturation is yet to be established (Zhang *et al.* 2010). It has been reported previously that bFGF treatment during maturation improves the development competence of bovine oocytes resulting in improved blastocyst development (Zhang and Ealy 2012). The theca and granulosa cells produce FGF2 and its transcript abundance increases during periovulatory follicle growth and during IVM (Valerio *et al.* 2010). Further, selective polymorphism in FGF2 gene has been linked with IVP success rate in bovine (Wang *et al.* 2009). Reduced developmental competence with oocytes derived from small follicles was reported (Yang *et al.* 2016).

In the current study, efforts were made to improve the *in vitro* development competence of small caprine oocyte through GDF-9 and bFGF treatments during the window of IVM. It is established that small oocytes exhibit poor development competence as compared to their larger counterpart. A large number of small follicles of various sizes are usually present in mammalian ovaries, but only a small number of them ultimately grow, attain full size, matured and ovulated. The growing oocytes synthesize and accumulate specific proteins and mRNA required for resumption of meiosis, maturation and post-fertilization development. The remaining non-growing small oocytes perish under *in vivo* condition and exhibit poor development competence if matured, fertilized and cultured *in vitro* (Su *et al.* 2014). It has been demonstrated in porcine that the presence of inhibiting activity in the ooplasm of small oocytes prevents nuclear maturation of large porcine and mouse oocytes fused to them (Fulka *et al.* 1985). In the current study, it was evident that the IVM rate of the small oocytes significantly improved with 30 ng/ml of GDF-9 as well as 20 and 30 ng/ml of bFGF supplementations. The results indicated that GDF-9 and bFGF supplementations at the specified level was probably effective in promoting favourable physiological and molecular changes in the small oocytes during IVM that resulted in better maturation rate. Further, post-fertilization development of the small oocytes following their exposure to the treatment of GDF-9 (30 ng/ml) and bFGF (20 ng/ml) combination during IVM was assessed. It was evident that the effect of the treatment was significant and it increased post-fertilization cleavage and morulae/blastocyst rates by 21 and 55% respectively, as compared to control. The results indicated that the treatment conferred better development competence to the small caprine oocytes, which they probably acquired during IVM and it was important for their post-fertilization development into advanced embryonic stage.

The current study demonstrated that the supplementation of GDF-9 (30 ng/ml) or bFGF (20 ng/ml) in IVM medium significantly improved the maturation rate of small caprine oocytes. Further, the supplementation of the combination of these growth factors in IVM medium significantly

improved the post-fertilization embryo development of small caprine oocytes.

ACKNOWLEDGEMENT

We are grateful to the Director, ICAR-NIANP, Bengaluru for providing necessary facility to carry out the research work. We also like to thank to Mr Shiv Kumar Tripathi for his help in preparation of the manuscript. Financial help from Department of Science and Technology, Govt. of India and JSPS, Govt. of Japan is gratefully acknowledged.

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