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Effect of GDF-9 and bFGF in combination on caprine granulosa cell growth parameters *in vitro*

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Granulosa cells are steroid secreting and hormone responsive cells under the influence of gonadotrophins. Therefore granulosa cells are mainly employed for studying steroidogenesis and oocyte/embryo culture. The role of different local intrafollicular growth factors on ovarian somatic cell growth has been an active research in recent years. The present study was undertaken to examine the effect of growth differentiation factor- 9 (GDF-9) and basic-fibroblast growth factor (bFGF) on *in vitro* growth of granulosa cells in goats.

Goat ovaries of unknown reproductive status were collected and brought to the laboratory within 2 h in a thermoflask containing warm normal saline from nearby corporation slaughter house, Bengaluru. Ovaries (n=642) were collected between September 2012 and May 2013. After aspirating the follicular fluid, cumulus oocyte complexes were picked up. The light brown sheets of compact granulosa cells (Nandi *et al.* 2008) were picked up, suspended in TCM-199 supplemented with 0.3% BSA, centrifuged at 500 g for 5 min. The cells were then washed for 2 times in washing medium (TCM-199 + 0.3% BSA), then the final pellet of granulosa cells was suspended in the medium in which they were to be cultured.

The chemicals used were procured commercial. In the preliminary studies, granulosa cells were cultured in media containing GDF-9 [0,10,20,30 ng/ml] or bFGF [0, 10, 20, 30 ng/ml]. The control medium consisted of TCM-199+bovine serum albumin (1%)+insulin-transferrin-selenium (1%) + gentamicin (50 mg/ml). The granulosa cell growth was higher in media containing GDF-9 and bFGF at the level of 20 ng/ml. Hence, in our main experiment, the granulosa cells (1×10^5/droplet) were cultured for 2 days, harvested and counted in a haemocytometer. The viability of the cells after culture was determined by the trypan blue exclusion test. In another experiment, the granulosa cells (1×10^5/droplet), were cultured in a 100 µl droplet of culture medium. The cells were cultured for 5 days; media were refreshed once on day 2 of culture. The monolayer formation in GC were evaluated for 5 day and scored as per Nandi *et al*. (2008). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays of the cells was measured as per Rooke (2004). The granulosa cell monolayer formation and maturation of oocytes on granulosa cell monolayer was done as described by Nandi *et al.* (2001) and Singh *et al*. (2010).

The statistical software "Graph Pad Prism" was used for analyzing the data. Maturation rate with different growth factors were analyzed by unpaired 't' test (the percentage values were transformed to arcsine values before analysis).

The effect of GDF-9 and bFGF in combination is presented in Table 1. Granulosa cell number increment and monolayer formation score were significantly higher ($P<0.05$) in media containing GDF-9 and bFGF at 20ng/ml. No significant difference was observed in viability, MTT oxidation and ability of granulosa cell monolayer to support oocyte maturation between control and treatment groups.

Granulosa cells, derived from mesonephric cells during embryogenesis increased in number during follicle growth by mitosis, possibly in response to FSH, estrogen and intraovarian peptides. With the accumulation of follicular fluid in the antrum, the granulosa cells in the follicles were

Table 1. Effect of incorporation of GDF-9 and bFGF in combination on granulosa cell growth parameters

Parameters	Control	GDF-9+bFGF
Viability (%)-2 d culture	94.2±2.21	95.1±2.65
Granulosa cell number increment- 2 d culture	$1.26\times10^5\pm0.02^a$	$1.65\times10^5\pm0.07^b$
Granulosa cell Monolayer-5 d culture	1.62 ± 0.18^a	2.50 ± 0.18^b
MTT oxidation -2 d culture-absorbance units (AU)/10^5 cells	0.142	0.148
Support of oocyte maturation (%)	78.4±1.22	84.2±1.27

Values in a row without any common superscripts differ significantly ($P<0.05$).

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separated into a membrana granulosa (mural granulosa cells) lying adjacent to the basement lamina and several to many concentric layers forming the cumulus oophorus around the oocyte (polar granulosa cells) (Wu *et al.* 2002). During growth, both *in vivo* and *in vitro,* the granulosa cells undergone cytological and biochemical changes which were related to the synthesis of various proteins and glycoproteins and to the production of steroids.

As the significance of these putative intraovarian regulators became increasingly recognized, much attention had been focused on the role of the local peptide factors. These factors are among the most potent stimulators of granulosa cell growth *in vivo* and *in vitro* (Knight and Glister 2001). FGFs influenced granulosa and theca cell function and follicular angiogenesis (Armstrong *et al.* 1997). However, the mechanism of action of FGFs in ovarian follicle cells is largely unknown (Jiang *et al.* 2012). GDF-9 promoted granulosa cell proliferation and prevent premature differentiation of the granulosa cells (Spicer *et al.* 2006). GDF-9 induced a signal transduction pathway which appears to be required for progesterone synthesis in cumulus granulosa cells (Elvin *et al.* 2000).

In conclusions, GDF-9 and FGF at 20 ng/ml improved granulosa cell functions and oocyte maturation indicating its influence on the mechanism of oocyte growth. The present study would help to unravel the role of GDF-9 and bFGF in oocyte growth/maturation that would pave way for understanding the mechanism of oocyte growth/maturation.

SUMMARY

The present study was undertaken to examine the effect of GDF-9 and bFGF on *in vitro* growth of granulosa cells in goats. Granulosa cell number increment and monolayer formation score were significantly higher in media containing GDF-9 and bFGF at 20 ng/ml. No significant difference was observed in viability, MTT oxidation and ability of granulosa cell monolayer to support oocyte maturation between control and treatment groups. In conclusions, GDF-9 and FGF at 20 ng/ml improved granulosa cell functions and oocyte maturation indicating its influence on the mechanism of oocyte growth.

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