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Expression of caspase-3, caspase-9, GDF-9 and IGF-1 genes in ovine oocytes cultured with optimum and elevated doses of Amphiregulin, Neuregulin-1 and Tumor necrosis factor-α *in vitro*

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ABSTRACT

The present study was conducted to evaluate the expression of caspase-3, caspase-9, growth differentiation factor-9 (GDF-9) and insulin growth factor-1 (IGF-1) genes in oocytes cultured *in vitro* with optimum and elevated doses of amphiregulin (50 ng and 150 ng), neuregulin-1 (25 ng and 150 ng) and tumor necrosis factor-α **(**25 ng and 150 ng) during *in vitro* maturation of oocytes based on the results of effects of AREG or NRG-1 or TNF-α concentration on maturation of oocytes which caused significant effect, were selected for gene expression studies along with the basal/control level. Total RNA was extracted from *in vitro* matured oocytes using Trizol method and Real-time reverse transcription polymerase chain reaction was used to evaluate the expression of genes. There was an upregulation of caspase-3 at 150 ng of TNF-α, caspase-9 at 50 ng of AREG, GDF-9 at 150 ng of AREG and IGF-1 at 150 ng of AREG and 25 ng of TNF-α. These results suggested that AREG at elevated dose and TNF-α at optimum dose enhanced the expression of GDF-9 and IGF-1, while the presence of elevated dose of TNF-α and optimum dose of AREG activated caspase-3 and caspase-9, respectively in oocytes cultured *in vitro*.

Keywords: Caspase-3, Caspase-9, GDF-9, IGF-1, Oocytes

The development of competent oocytes is dependent on communication between oocytes and cumulus cells, as well as on cumulus cells for the glycolysis and cholesterol biosynthesis pathways, which are not present in oocytes (Huang and Wells 2010). Richani *et al*. (2013) found that Amphiregulin (AREG) acting on both mural granulosa and cumulus cells, improved oocyte maturation and developmental competence. Similarly, Park *et al.* (2004) expressed that treatment with AREG induced cumulus expansion, oocytes maturation and COX-2 expression in mouse and rodent follicles which induced re-initiation of vesicle meiosis called germinal vesicle breakdown (GVBD). According to Noma *et al*. (2011), NRG-1 stimulated particularly AKT/PKB phosphorylation in cultured granulosa cells and regulated luteinization, oocyte maturation, and ovulation through autocrine and paracrine manner. On the other hand, Field *et al.* (2014) reported that granulosa cells, theca cells and oocytes produce TNF-α which is necessary for GC proliferation, oocytes apoptosis, follicular apoptosis and atresia. Furthermore, Fenwick and Hurst (2002) opined that caspase-3 required proteolytic cleavage for its activation and is a key mediator of apoptosis. Whereas, Robles *et al.* (2000) reported that caspase-9 initiated caspase-3 activities in the antral follicles. The growth factor GDF-9 played an important role in the development of follicles by upregulating theca cell and androgen production (Orisaka *et al*. 2009). GDF-9 promoted pre-antral to early antral transition of the follicle and improved blastocyst development and also Inner cell mass (ICM) cell numbers.

The present study was conducted to evaluate the expression of caspase-3, caspase-9, growth differentiation factor-9 (GDF-9) and insulin growth factor-1 (IGF-1) genes in oocytes cultured *in vitro* with optimum and elevated doses of amphiregulin, neuregulin-1 and tumor necrosis factor-α during *in vitro* maturation of oocytes.

MATERIALS AND METHODS

Collection and evaluation of oocytes from surface antral follicles [Small antral follicles (SAFs), Medium antral follicles (MAFs), Large antral follicles (LAFs)]: The experiments were conducted both in Veterinary College, Bengaluru and ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru. Sheep ovaries from

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a civil slaughterhouse, Bengaluru were transferred to the laboratory in a thermo-flask at 38°C and then washed and stored in physiological saline at 38.5°C. The number of visible follicles were classified in each ovary into: SAFs (< 1 mm), MAFs (1-4 mm) and LAFs (> 4 mm) groups (Bari *et al.* 2011). The oocytes were aspirated from these different sized follicles using a 20-gauge hypodermic needle attached to a 5 ml syringe containing 0.5-1.0 ml of aspiration medium and pooled in a test tube and allowed to sediment for 10 min. The sediment was taken and washed twice with PBS, then it was placed in a petridish and the oocytes were collected by a pipette. The oocytes were checked for viability using the trypan blue staining technique and were washed thrice in TCM-199 supplemented with 0.3% BSA. Only oocytes surrounded by compact multi-layered cumulus with greater than 3 layers of cumulus cells and a homogeneous ooplasm were selected for *in vitro* maturation of oocytes (Nandi *et al.* 2006).

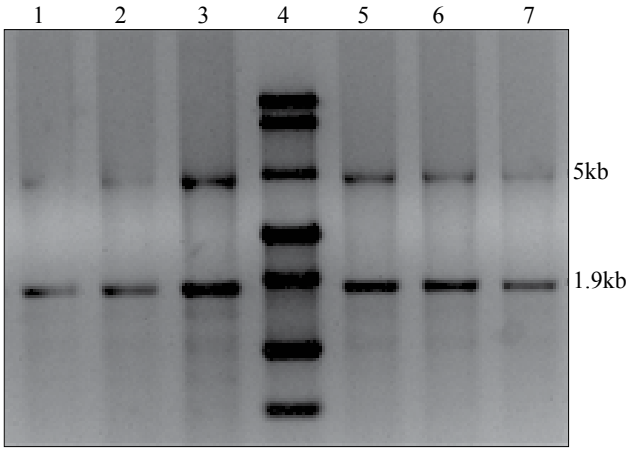
In vitro *maturation of oocytes:* Oocytes were washed once with the aspiration medium and twice in the medium in which they were to be cultured. Oocytes were transferred in Groups (5-8/Group) into 50 µl droplets of IVM culture medium in a 35 mm petridish consisting of TCM-199, 10% fetal bovine serum (FBS), 10 µg/ml of follicle stimulating hormone (FSH) and 50 µg/ml of gentamicin. The droplets containing oocytes were then covered with warm (38.5°C) mineral oil and the petridishes were placed in a CO_2 incubator (38°C, 5% CO_2 in air, 90-95% relative humidity) for 24 h.

Assessment of oocytes maturation: Cumulus expansion was observed by stereo-zoom microscope and was assessed at 24 h of IVM according to the visual assessment of the degree of expansion (cumulus expansion score). Degree-0: no expansion; Degree-1: moderate expansion, cumulus cells were non-homogeneously spread and clustered cells were still observed and Degree-2: fully expanded, CCs were homogeneously spread and clustered cells were no longer present. The parameters of oocytes maturation was extrusion of a first polar body in the perivitelline space and expansion of cumulus cell layer-Degree 2 and Degree 3 (Nandi *et al.* 2002). The oocytes viability was determined by trypan blue staining technique and was in the range of 85-95%.

Gene expression studies: Based on the results of levels of AREG or NRG-1 or TNF-α and their combinations on maturation of oocytes which caused the significant effect (Ramesh *et al.* 2021) the levels of the growth factors were selected for gene expression studies along with the basal/ control level. *In vitro* matured oocytes were lysed in 300 µl of RTL buffer containing 10 µl/ml of 2-mercaptoethanol and stored at -80°C until RNA extraction.

RNA extraction: Total RNA was extracted from each frozen sample of *in vitro* matured oocytes, using Trizol method. The absorbance values at 260 nm and 280 nm were checked to assess the RNA concentration and purity of protein impurities in the samples. The purity and integrity of the RNA was again checked by using native agarose gel electrophoresis (Fig. 1).

Primer design and synthesis: Gene specific primers (Table 1) were designed using online NCBI primer



Lane 1: AREG (50 ng)
Lane 2: AREG (150 ng)
Lane 3: NRG1 (25 ng)
Lane 4: Ladder
Lane 5: NRG1 (150 ng)
Lane 6: TNFα (25 ng)
Lane 7: TNFα (150 ng)

Fig. 1. RNA isolated from the *in vitro* matured oocytes of different media formulation run on native agarose gel electrophoresis.

Table 1. List of primers used

Gene Name	Primer	Primer sequence (5'- - - - - - - caspase-3')	Gene Bank accession No.
GAPDH	F	GGGTCATCATCTCTGCACCT	NM_001190390.1
	R	GGTCATAAGTCCCTCCACGA	
IGF-1	F	GCTCTCAACATCTCCCATCTCC	NM_001009774.3
	R	GACTGGCATCTTCACCTGCTTC	
GDF-9	F	CTTTGCCTGGCTCTGTTTTC	NM_001142888.2
	R	TCCCACCTAAATGGTTCAGC	
Caspase-3	F	TAGCAAGTTTCTTCAGAGGG	Ebrahimi *et al.*(2010)
	R	GTCTCAATACCACAGTCCAG	
Caspase-9	F	GCCCTTGCCTCTGAGTAGTG	Wei *et al.*, (2017)
	R	CCAACCAAATGAAGCCAAGT	

design software (Primer 3, http://bioinfo.ut.ee/primer 3/) (Thornton and Basu 2011). Specificity was checked using Primer 3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Real-time reverse transcription polymerase chain reaction: The total RNA was reverse transcribed using a reverse transcription level kit (iscript cDNA synthesis kit, California, USA) according to the manufacturer protocol. First-strand cDNA of matured oocytes obtained after reverse transcription was used for carrying out PCR using specific primers for caspase-3, caspase-9, GDF-9 and IGF-1 and its receptors and house-keeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). GAPDH was used as the endogenous control. Quantitative real-time PCR analysis was performed with KAPA SYBR FAST qPCR Master mix (2×) (Kapa Biosystems, United States Wilmington, Massachusetts). Each run was performed in duplicate in a 20 µl reaction that contained 10 µl qPCR master mix, 5 µM of 0.5 µl gene-specific forward and reverse primers, 2 µl of cDNA as template and final volume of 20 µl was made up with nuclease-free water. The PCR conditions used to amplify all genes includeed, initial denaturation at 95°C for 3 min with 40 cycles of denaturation at 95°C for 30 sec followed by annealing and extension at 60°C for 30 sec. The gene expression levels of matured oocytes were analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis: Analysis was done by using software SPSS-17 (Online trial version). The changes in relative expression of different genes in relation to the GAPDH, a reference house-keeping gene were calculated as per Livak and Schmittgen (2001). Differences between the mean values were considered significant when the P values less than 0.05.

RESULTS AND DISSCUSSION

Maturation rate of oocytes was significantly higher ($p<0.05$) in groups exposed to optimum and elevated doses of amphiregulin (50 ng and 150 ng), neuregulin-1 (25 ng and 150 ng) and optimum dose of tumor necrosis factor-α (25 ng) (Ramesh *et al*. 2021). RNA quality and integrity was checked by agarose gel electrophoresis as shown in Fig. 1.

Effect of AREG, NRG-1 and TNF-α on relative mRNA expression of caspase-3 during in vitro *maturation of oocytes:* The RNA extracted from all oocytes cultured with AREG (50 ng and 150 ng), NRG-1 (25 ng and 150 ng) and TNF-α (25 ng and 150 ng) when subjected to relative expression of caspase-3 revealed 0.8 fold higher expression in oocytes treated with 50 ng of AREG when compared with the 150 ng of AREG (Table 2). There was no significant change in 25 ng and 150 ng NRG-1 when compared with 50 ng AREG. However, significant change (0.9 and 0.7) in expression was observed in 25 ng NRG-1 and 150 ng NRG-1 compared with 150 ng AREG, respectively. Further, there was no significant change in expression observed in 50 ng AREG and 150 ng TNF-α. The present study indicates the TNF-α induced apoptosis in granulosa cells through the caspase-3 and Bcl- 2 family members, which was in agreement with an earlier report (Yamamoto *et al.* 2015). Furthermore, Prange-Kiel *et al.* (2001) reported that TNF-α is said to have influenced the balance between follicular growth (proliferation) and atresia (apoptosis). On the contrary, Yamamoto *et al.* (2015) reported that cleaved caspase-3 which is the final executioner in apoptotic cell death was significantly decreased after addition of TNF-α. At the higher dose (100 ng/ml), NRG-1 co-treated GCs showing significantly lower expression of active caspase-3 when compared with lower-dose (10 ng/ml) NRG-1 co-treated groups (Chowdhury *et al*. 2017).

Effect of AREG, NRG-1 and TNF-α on relative mRNA expression of caspase-9 during in vitro *maturation of oocytes:* The RNA extracted from all oocytes cultured with AREG (50 ng and 150 ng), NRG-1 (25 ng and 150 ng) and TNF-α (25 ng and 150 ng) when subjected to relative expression of caspase-9 revealed no significant fold changes in expression in oocytes treated with 150 ng of AREG, 25 ng of NRG-1, 150 ng of NRG-1 and 25 ng of TNF-α. However, there was a significantly higher expression changes observed in 50 ng of AREG when compared with all other doses.The observation made in this study supported the finding of Mao *et al.* (2004) wherein AREG inhibited apoptosis and caused granulosa cell proliferation. Based on these findings, it can be assumed that oocytes cultured with AREG have increased oocyte developmental competence (Richani *et al.* 2013).

Effect of AREG, NRG-1 and TNF-α on relative mRNA expression of GDF-9 during in vitro *maturation of oocytes:*

Table 2. Effect of AREG (50 ng and 150 ng), NRG-1 (25 ng and 150 ng) and TNF-α (25 ng and 150 ng) on relative mRNA expression of different genes during *in vitro* maturation of oocytes

Treatment	Relative mRNA expression of different genes			
	Caspase-3	Caspase-9	GDF-9	IGF-I
Control	1.0±0.02	1.0±0.03	1.0±0.01	1.0±0.03
AREG (50 ng)	1.8±0.05^{bc}	1.9±0.09^{a}	3.9±0.04^{abc}	4.7±0.04^{ab}
AREG (150 ng)	1.0±0.04^{a}	1.2±0.02^{b}	5.2±0.03^{a}	5.7±0.07^{b}
NRG-1 (25 ng)	1.9±0.05^{bc}	1.4±0.06^{b}	3.0±0.05^{b}	3.8±0.02^{a}
NRG-1 (150 ng)	1.7±0.09^{abc}	1.5±0.05^{ab}	3.1±0.08^{b}	3.8±0.09^{a}
TNF-α (25 ng)	1.2±0.07^{ab}	1.3±0.02^{b}	4.5±0.05^{ac}	5.6±0.05^{ab}
TNF-α (150 ng)	2.2±0.02^{c}	1.6±0.04^{ab}	3.3±0.03^{bc}	4.0±0.04^{ab}

Superscripts bearing different letters in the same column differ significantly ($p<0.05$).

The RNA extracted from all oocytes cultured with AREG (50 ng and 150 ng), NRG-1 (25 ng and 150 ng) and TNF-α (25 ng and 150 ng) when subjected to relative expression of GDF-9, showed significant higher fold change (2 fold) in expression observed in the 150 ng of AREG compared to 25 ng NRG-1, 150 ng NRG-1 and 150 ng TNF-α (Table 2). This might be due to AREG stimulating oocytes by activating GDF-9, causing ID1 and ID3 transcription factors to be expressed in the granulosa cells of ovine follicles, which is required for oocyte development as suggested by Sugimura *et al.* (2014) and Milan Blaha *et al.* (2015). This could also be due to GDF-9 increasing the activity of M-phase-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) in oocytes, resulting in better oocyte quality and eventual developmental potential *in vitro* (Lin *et al*. 2014). According to Sugimura *et al.* (2015) AREG in combination with GDF-9 is required to enhance oocyte meiotic maturation and blastocyst formation. The observation made in his study supports the findings of Yan *et al.* (2001) wherein GDF-9 plays a key role in enhancing development of granulosa cell and oocytes-cumulus cell products. Our findings were also supported by Gode *et al*. (2011) wherein higher levels of GDF-9 in the follicular fluid were linked to oocyte maturation and embryo quality. Furthermore, oocyte maturation, fertilization rate, and cleavage rate were significantly higher in the group with higher GDF-9 mRNA expression than in the group with lower GDF-9 mRNA expression, where oocyte maturation was positively related to GDF-9 mRNA expression as has been opined by Li *et al*. (2014). According to Sudiman *et al*. (2014), GDF-9 has been demonstrated to enhance the development of oocytes in animal experiments. Based on these findings, it can be assumed that GDF-9 stimulates the proliferation and metabolism of granulosa cells, which in turn stimulates the expression of kit ligand (KL) on granulosa cells, which then acts on its receptor on the oocytes, resulting in oocyte development and maturation (Gilchrist *et al*. 2008).

Effect of AREG, NRG-1 and TNF-α on relative mRNA expression of IGF-1 during in vitro *maturation of oocytes:* The RNA extracted from all oocytes cultured with AREG (50 ng and 150 ng), NRG-1 (25 ng and 150 ng) and TNF-α (25 ng and 150 ng) when subjected to relative expression of IGF-1, showed significantly higher fold change in expression observed in 150 ng of AREG and 25 ng of TNF-α compared to 25 ng NRG-1, 150 ng NRG-1 and 150 ng TNF-α (Table 2). This could be due to coordination of IGF-1 and EGF-like growth factors such as AREG which stimulated MAPK signaling (Park *et al*. 2004). Our data showed that 150 ng AREG increased the expression of IGF-1 genes. The observation made in this study supported the finding of Jamnongjit and Hammes (2006) wherein IGF-1 promoted oocytes maturation in vitro. According to Frank (2014) maturation, fertilization rate and embryo development were increased in the presence of IGF-1.

Our results showed that the addition of elevated dose of AREG to the culture media can induce oocytes maturation as evidenced by the activation of GDF-9 and IGF-1. The present results also revealed that addition of elevated dose of TNF-α and optimum dose of AREG could induce apoptosis as evidenced by activation of caspase-3 and caspase-9, respectively. These findings suggest that detecting GDF-9 and IGF-1 mRNAs in cumulus cells could be useful as invasive biomarkers for assessing oocyte developmental potential, as well as caspase-3 and caspase-9 for assessing apoptosis.

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