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Effect of Leptin and IGF-I Supplementation on *in vitro* Maturation and Expression of Apoptotic Genes in Goat Oocytes

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ABSTRACT

Background: The quality of gametes is a crucial factor for the successful development of an embryo. The potential of oocyte development is acquired during oogenesis by many changes at the molecular and cellular levels. The present study explores the effect of leptin and IGF-I supplementation on *in vitro* oocyte maturation and the expression of apoptotic genes in matured goat oocytes.

Methods: *In vitro* maturation of abattoir-derived oocytes was in maturation media having supplements as the group I (control), group II (leptin 20 ng/ml) and group III (IGF-I 100 ng/ml). From these oocytes, RNA was isolated and expressions of apoptotic genes were studied.

Result: There was no significant difference found between cumulus expansion and also in nuclear maturation in groups I, II, III. The relative expression of *BAX* gene was 0.65 fold lower in group II and 0.42 fold lower in group III as compared to group I whereas relative expression of *Mcl-1* gene was 2.55 fold higher in group II and 0.31 fold lower in group III as compared to group I.

Key words: Apoptotic gene, Goat oocytes, IGF-I, *In vitro* maturation, Leptin.

INTRODUCTION

For successful embryo development, quality of gametes is a pivotal factor. Several changes at the molecular and cellular levels occur during oogenesis to gain developmental potential. These changes enable the oocyte to accomplish meiosis, monospermic fertilization and transition through early developmental stages until embryonic genome activation (Coticchio *et al.,* 2004). Microenvironment during *in vitro* culture of pre-implant embryos is important for simulating their developmental potential that depends on the presence of regulatory proteins, growth factors and hormones in the culture media (Kaya *et al*., 2017). Different culture conditions affect gene expression in oocyte and pre-implanted embryos (Wrenzycki *et al.,* 2001). During the pre-implantation development of the embryo, apoptosis is regulated by pro and anti-apoptotic genes (Exley *et al.,* 1999). Members of *Bcl-2* gene family of which there are at least 15 mammalian *Bcl-2* gene family members, play a key role in the regulation of apoptosis and categorized into two subgroups, anti-apoptotic (*Bcl-2, Bcl-w, Bcl-x$_l$, A1, Mcl-1*) and pro-apoptotic (*Bax, Bik, Blk, Hrk, BNIP$_3$, Bcl-x$_s$*) (Cory and Adam, 1998). *Bax* gene is known to reside in the cytoplasm or cell membrane, whose cytoplasmic elevation causes induction of oocyte apoptosis (Morita and Tilly, 1999). *Bcl-2* protein is found in the nuclear envelope and mitochondria that promotes germ cell survival (Flaws *et al*., 2001).

Leptin is a 16-kDa cytokine that plays an important role in reproduction through oocyte maturation and developmental competence in cattle (Boelhauve *et al.,* 2005, Jia *et al.,* 2012) and buffalo (Panda *et al*., 2017). Leptin improves maturation rate, cleavage, morula and blastocyst development in buffalo oocytes (Singh *et al.,* 2012). IGF-I plays an essential role in mammalian reproduction (Kadakia *et al*., 2001), affects cell growth, differentiation and has anti-apoptotic effects during *in vitro* embryo development (Stefanello *et al.,* 2006). Also, IGF-I has been implicated in at different stages of follicular development and oocyte maturation (Demeestere *et al.,* 2004). Thus, the present study has been designed to explore the effect of leptin and IGF-I supplementation in maturation media for assessing their effect on *in vitro* maturation and expression of pro-apoptotic *BAX* and anti-apoptotic *Mcl-1* genes of abattoir derived goat oocytes.

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MATERIALS AND METHODS

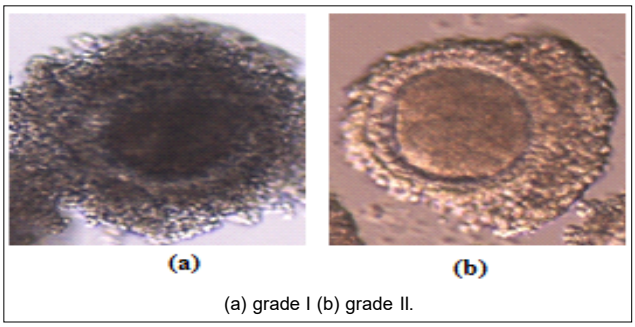
The experiment was conducted at Animal Biotechnology Centre, Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India in the year 2019.

Oocyte collection and grading

The goat ovaries were procured from Small Animal Abattoir, Jabalpur and were transported to the laboratory in warm normal saline (30-35°C). The ovaries were trimmed and washed with Dulbecco's phosphate buffer saline (DPBS) followed by brief exposure to 70% ethanol and finally rinsed with DPBS. The oocytes were aspirated from 2-8 mm sized ovarian follicles through the syringe aspiration method. The oocytes were washed and transferred to maturation media. Grading of oocytes was done on the basis of layers of cumulus cells and homogeneous granular grey cytoplasm, under a stereo-zoom microscope (Kumar *et al.,* 2016).

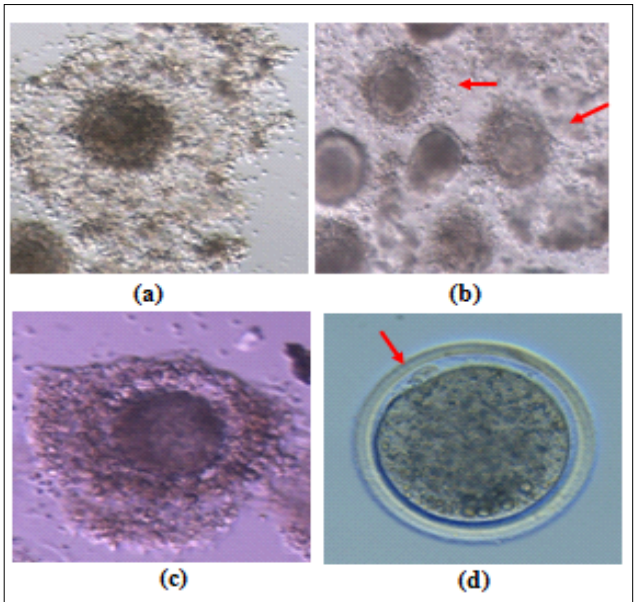
In vitro maturation of oocytes

The retrieved grade I and grade II (Fig 1) quality of cumulus-oocyte complex (COCs) were kept in maturation medium [TCM-199 (Hyclone medium 199/EBSS, Cat#SH30253.01), 7.5% v/v FBS (Hyclone, Cat# SH30070.02), 10 µg/ml follicle stimulating hormone (Sigma, Cat#F2293),10 µg/ml luteinizing hormone (Sigma, Cat#F5269), 1 µl/ml oestradiol (Sigma, Cat#E8875), sodium pyruvate (0.8 mM/ml) and gentamicin (50 µg/ml)] in groups of 25-30 oocytes per droplet. The droplets were then covered with sterile pre-equilibrated mineral oil and incubated in a CO_2 incubator with> 90% relative humidity and 5% CO_2 tension at 38.5°C for 27 hrs. Oocytes were divided into three groups and subjected to the following supplementation in maturation media-Group I (control; no supplements), Group II (Leptin 20 ng/ml) and Group III (IGF-I 100 ng/ml).



(a) grade I (b) grade II.

Fig 1: Grades of immature goat oocytes obtained from abattoir-derived ovaries.



(a) grade A (b) grade B (c) grade C; (d) An oocyte showing first polar body.

Fig 2: Grades of cumulus expansion of matured goat oocytes.

The assessment of cytoplasmic maturation of matured oocytes was done by visualization of the degree of the cumulus expansion and graded as grades A, B and C (Hunter and Moor, 1987) (Fig 2). For nuclear maturation assessment, the cumulus cells from matured oocytes were removed by 0.1% hyaluronidase. The denuded oocytes were observed under an inverted stereo-zoom microscope and it showed one extruded polar body between the peri-vitelline membrane and zona pellucida (Fig 2d). Then 50 oocytes from each of the three groups were kept in 1 ml Trizol reagent at -20°C until use, for quantitative real-time expression of apoptotic genes.

Quantitative real-time expression of apoptotic genes in matured oocytes

Total RNAs from matured oocytes were isolated by the Trizol method as per the protocol described in molecular cloning (Jeena, 2020) with some modifications. The concentration of total RNA was determined by Nanodrop™ ND-2000 spectrophotometer (OD at 260). The total RNA samples were treated with DNase I (Fermentas) for removal of possible genomic DNA contamination. From the extracted RNA, cDNA was synthesized using the Revert Aid™ first-strand cDNA Synthesis kit (Thermo Fisher Scientific). Description of forward (F) and reverse (R) primers used to assess the expression (Sambrook and Russell, 2001) of selected genes (Table 1). The Real-time PCR (ABI Prism 7500) was performed by SYBR green chemistry (ABI, USA) using cDNA as a template. All PCR reaction was performed in triplicates with *β-actin* as an internal control. The reactions were carried out in an optical 96 wells plate with a final reaction volume of 20 µl using 1 pmol/µl primers (forward and reverse) for each gene. The specificity of amplification during real-time PCR was monitored by evaluating the melting curve and running the PCR products on 1.8% agarose gel (Fig 3b). The real-time PCR was optimized with respect to primer concentration and annealing temperature. The amplification efficiency of the genes were compared by two-fold dilution for *BAX* and *Mcl-1* genes from reference cDNA samples. The samples were then amplified in real-time RT-PCR and the cycle threshold (C_T) values were normalized to *β–actin* (housekeeping gene). The comparative quantification of *BAX* and *Mcl-1* gene in mRNA isolated from different treatment groups was done by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Quantification data were analyzed with the Sequence Detection System (SDS) software v1.3.0 (Applied Biosystems).

RESULTS AND DISCUSSION

In vitro maturation and cumulus expansion of oocytes

The COCs obtained from abattoir derived ovaries are variable in their developmental competence and depends on the presence of cumulus cell layers (Bilodeau-Goeseels and Panich, 2002), presence of adequate growth factors, hormones and regulatory proteins in the oocyte maturation media as well as the embryo culture media. For the optimum development of oocytes, their fertilization and embryonic development, cumulus expansion plays an important role (Han *et al*., 2006 and Kumar *et al*., 2016). The expansion of cumulus cell layers promotes the fertilization by loosening the barrier for sperm penetration. The cumulus cells secrete some factors into the culture media that may facilitate gap junction communication between cumulus cells and oocytes, which are essential for optimum fertilization. Higher cumulus expansion of oocytes has also been correlated with the higher percentage of polar body extrusion as compared to oocytes having less expanded cumulus cells (Kumar *et al*., 2016).

The present study comprised of 15 replicates including 476 ovaries from which 1581 COCs of grade I and grade II were subjected to *in vitro* maturation. The cumulus expansion of matured oocytes were grade A- 45.81±0.14, 47.76±0.14, 47.37±0.34 per cent; grade B- 37.01±0.11, 38.39±0.17, 38.20±0.30 per cent and grade C- 17.18±0.11, 13.86±0.08, 14.16±0.31 per cent in groups I, II, III respectively (Table 2). Hence the addition of leptin and IGF-I, non-significantly increased the percentage of cumulus expansion (P>0.05).

Nuclear maturation

This is characterized by the oocyte's ability to resume meiotic division up to metaphase II during *in vitro* maturation. It is visualized by the extrusion of the first polar body (Pursel *et al.,* 1985) (Fig 2d). In the present work, the nuclear maturation of matured oocytes was recorded as 93.43±1.59, 97.55±0.40 and 94.46±0.91 per cent between groups I, II and III respectively. No significant increase in the percentage of nuclear maturation was found on addition of leptin and IGF-I (Table 2). However, Khaki *et al*. (2014) reported the addition of leptin (10 ng/ml) in maturation media of buffalo oocyte, increased first polar body extrusion (83.81% *vs*. 74.65% in control). A higher percentage of the first polar body was recorded by adding 10 ng/ml leptin in the serum-free maturation media (Paula-Lopes *et al.,* 2007).

Table 1: Description of primers used for gene expression.

Primer	Primer sequence (5′-3′)	Primer size	Amplification size
BAX (F)	ATCCACCAAGAAGCTGAG	18	83 bp
BAX (R)	CTGCGATCATCCTCTGTA	18	
Mcl-1 (F)	AGGCGGCATCAGAAATGTG	19	200 bp
Mcl-1 (R)	TCCATCGCTAGGTGATGGC	19	

Table 2: *In vitro* maturation of goat oocytes matured with leptin and IGF-I supplements.

Group	Ovary (n)	Oocytes (n)	Per cent cumulus expansion			Per cent nuclear maturation
			Grade A	Grade B	Grade C	
Group I	213	535	45.81±0.14	37.01±0.11	17.18±0.11	93.43±1.59
Group II	110	534	47.76±0.14	38.39±0.17	13.86±0.08	97.55±0.4
Group III	153	512	47.37±0.34	38.20±0.3	14.16±0.31	94.46±0.91

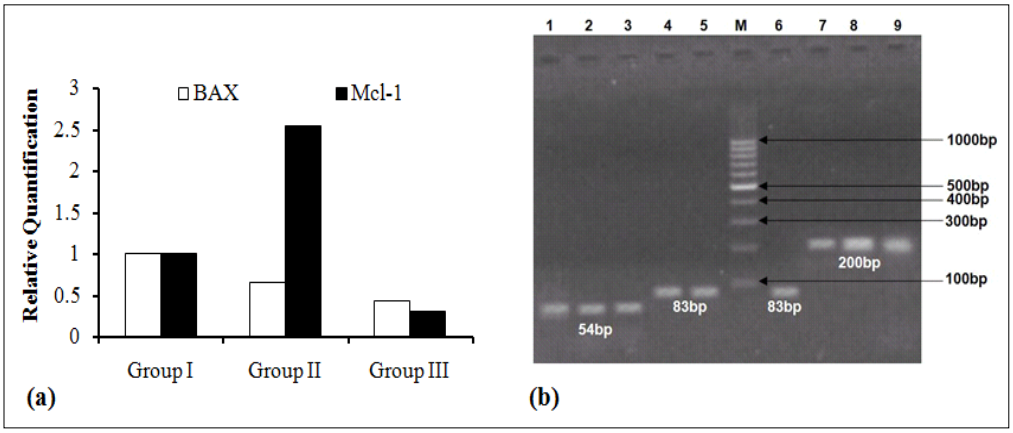


Fig 3: (a) Expression profiling of *BAX* and *Mcl-1* gene in mature goat oocytes (P≤0.1; based on chi-square analysis) (b) Agarose gel electrophoresis (1.8%) of RT-PCR products for *β-actin* (54 bp; Lane 1,2,3), *BAX* (83 bp; Lane 4,5,6) and *Mcl-1* (200 bp; Lane 7,8,9) genes of group I, II, III respectively. Lane M: 100 bp DNA ladder.

media increased nuclear maturation as reported by Lorenzo *et al*., (1994) (45.5% *vs.* 35.6% in control). The variation in nuclear maturation rate may be due to species variation.

Gene expression analysis of pro-apoptotic (*BAX*) and anti-apoptotic (*Mcl-1*) genes in matured oocytes

In the present experiment, two-fold serial dilutions of cDNA templates of each group were utilized for expression study of *BAX* and *Mcl-1* gene by RT-PCR. The primers of respective genes in all groups clearly showed amplification of all the genes by RT-PCR. The specificity of the reaction was confirmed by the single peak of dissociation curve amplification plot. Since the efficiencies of the target and endogenous control (β-*actin*) amplifications are approximately equal, thus data obtained from Real-time qPCR by $\Delta\Delta$CT method were used for calculating the relative expression for *BAX* and *Mcl-1* gene (Livak and Schmittgen, 2001).

In order to determine the relative expression study of each gene, the mRNA transcript level of group I without any supplement was taken as a referral control. The present study revealed that the relative expression of the *BAX* gene was 0.65 fold lower in Group II and 0.42 fold lower in Group III as compared to Group I. The relative expression of *Mcl-1* gene was 2.55 fold higher in Group II and 0.31 fold lower in Group III as compared to Group I (Fig 3a).

Morita and Tilly (1999) suggested that *BAX* expression is the marker of stress and predictor of cell fate. *Mcl-1* acts as an essential survival factor for growing follicle and effective mitochondrial function (Omari *et al*., 2015). Leptin increases $BIRC_4$ levels (baculoviral inhibitor of apoptosis protein repeat) in blastocysts derived from 1 ng/ml leptin treated oocytes and reduced pro-apoptotic-*BAX* mRNA levels in blastocysts derived from 10 ng/ml leptin treated oocytes (Boelhauve *et al*., 2005). The oocyte of good quality showed a high expression level for the *Bcl-2* gene and low for the *BAX* gene (Yuan *et al.,* 2002).

Both leptin and IGF-I supplementation in maturation media causes decreased expression of pro-apoptotic *BAX* gene in matured goat oocytes. However, expression of the *Mcl-1* gene in matured goat oocytes is enhanced by leptin and reduced by IGF-I when supplemented in maturation media.

CONCLUSION

In the present study, the addition of leptin and IGF-I to *in vitro* maturation media noticeably did not increase the oocyte maturation. Leptin supplementation in maturation media might be beneficial as it reduced the pro-apoptotic *BAX* gene and increased the anti-apoptotic *Mcl-1* gene expression in matured goat oocytes. IGF-I supplementation to the maturation media reduced the pro-apoptotic *BAX* gene and anti-apoptotic *Mcl-1* gene expression in matured goat oocytes. It requires more trials on pro-apoptotic and anti-apoptotic gene pathways to establish that IGF-I does not affect the maturation of goat oocytes.

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Conflict of interest

All authors declare that they have no conflict of interest.

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