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Evaluation of L-ascorbic acid and goat follicular fluid supplementation on *in-vitro* maturation of goat oocytes

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Abstract
This study was conducted to compare the effect of L-ascorbic acid and goat follicular fluid supplementation on *in-vitro* maturation of goat oocytes. In order to evaluate the effect of these two supplements, cumulus oocytes complexes (COCs) were collected from goat ovaries by aspiration, slicing and puncture method. Irrespective of oocyte retrieval techniques, grade-I oocytes (n=337) in 16 replicates were matured *in-vitro* in TCM-199 (with estradiol @ 1 µg/ml, eCG @ 20 IU/ml, hCG @ 20 IU/ml, 10% EGS (Estrus goat serum) and Gentamicin sulphate @ 50 µg/ml) as control that was supplemented with 50 µg/ml of L-ascorbic acid and 20% goat follicular fluid for 27 hours. Assessment of maturation was done based on the degree of cumulus cells expansion and extrusion of first polar body under stereo-zoom microscope. Significantly higher (p<0.05) proportion of oocytes were matured *in-vitro* in medium supplemented with goat follicular fluid compared to medium supplemented with L-ascorbic acid and control. It was concluded that enriching the maturation medium with goat follicular fluid has beneficial effect on *in-vitro* maturation of goat oocytes.

Keywords: Goat, *in-vitro* maturation, oocytes, L-ascorbic acid and goat follicular fluid

1. Introduction
Among all species of farm animals, Goats (*Capra hircus*) have the widest ecological range and have been poor people's most reliable livelihood resource popularly known as “poor man’s cow”. The fundamental area of raising animals is a reproduction by maximizing the use of tested sires and dams and using embryo transfer technology. Follicular oocytes could be matured *in-vitro* and used for *in-vitro* fertilization for producing a large number of embryos (Agarawal, 1992) [1]. In recent years, there has been an increase in interest in large-scale *in-vitro* production of goat embryos by *in-vitro* maturation, fertilization, and culture of oocytes for quicker multiplication of superior germplasm. *In-vitro* maturation (IVM) of oocytes is an Assisted Reproductive Technology (ART) that enables mature oocytes to be generated *ex vivo*. Cumulus-oocyte complexes (COCs) are artificially removed from antral follicles and cultured until they develop or reach the metaphase II (MII) stage under essentially standard cell culture conditions. (Gilchrist and Thompson, 2007) [5]. Supplementation of protein, follicular fluid, hormone, growth factor and antioxidants in the maturation medium has been reported to enhance the process of IVM, fertilization and subsequent embryo development. The maturation status of oocyte cytoplasm plays a major role in reprogramming gene expression at the onset of embryonic development (Rios *et al*., 2015) [12]. It becomes necessary that IVF protocols need to be revised to incorporate strategies to prevent ROS injuries. This could be achieved by supplementation of antioxidants in the culture media. Several substances are used to supplement culture media to improve oocyte maturation. The follicular fluid one of these substances is used (Asgharimoghadam *et al.*, 2015) [3], which provides such an environment where oocytes could nourish and undergo maturation like *in-vivo*. Understanding what constitutes oocyte developmental competence is one of the major problems that still face the sciences of reproductive and developmental biology. Though many studies have been carried out to improve the developmental potential of oocytes, the results are still inconsistent. Therefore, the present study aimed to investigate factors affecting IVM of goat oocytes, the effect of hormones, serum, antioxidant, and follicular fluid supplementation on maturation medium.

2. Materials and Methods

2.1 Collection and processing of ovaries

Goat ovaries were collected from local slaughterhouses as soon as possible after the animals were slaughtered in and around Bidar district (Karnataka) with an undefined stage of the reproductive cycle. Ovaries were collected in a thermos flask containing warm (35^{0}-37 °C) Normal Saline Solution (N.S.S.) fortified with 50 µg/mL gentamicin sulphate solution and transported to the laboratory within 2-3 hours. In the laboratory, extraneous tissues were removed from the ovaries and they were washed 4-5 times with N.S.S. containing antibiotic for further processing. The oocytes were recovered from the ovaries immediately after washing, examined under a stereo-zoom microscope. Only good quality (grade-I) oocytes surrounded by more than four layers of cumulus cells adhered to the zona pellucida were used for IVM.

2.2 Collection and preparation of follicular fluid

Goat follicular fluid collection was performed by aspiration of 2-6 mm diameter follicles using a syringe with needle size 18G, centrifuged at 3000 RPM for 10 minutes, filtered using Millipore 0.22µm, inactivated at a temperature of 56 °C for 30 min and stored in the freezer @ -20 °C until its use (Wahjuningsih *et al*., 2014) [13].

2.3 *In-vitro* maturation of oocytes

Three different treatment groups were made based on the hormones used in the basic maturation media prepared by using TCM-199, estradiol @ 1 µg/ml, eCG @ 20 IU/ml, hCG @ 20 IU/ml, 10% EGS (Estrus goat serum) and Gentamicin sulphate @ 50 µg/ml as control that was supplemented with 50 µg/ml of L-ascorbic acid and 20% goat follicular fluid. Only Grade-I oocytes were washed in maturation media 3-4 times. After washing 10-15 oocytes were cultured in droplets of pre-incubated maturation media and cultured at 38.5 °C, 5% CO_2, 90-95% RH for 27 hours in a CO_2 incubator.

2.4 Assessment of *in-vitro* maturation

2.4.1 Based on the degree of expansion

After 27 hours of maturation, an assessment of oocyte maturation was done based on the degree of cumulus expansion (Kobayashi *et al.*, 1994) [7] as Degree 0: No expansion, Degree 1: Cumulus cells were non-homogenously spread and clustered cells were still observed, Degree 2: Cumulus cells were homogenously spread and clustered cells were no longer present. Only degree 1 and 2 cumulus expanded oocytes were considered as matured ones and the maturation rate was calculated by dividing the total number of degree l and 2 matured oocytes by the total number of oocytes utilized for maturation by particular media.

2.4.2 Based on the extrusion of the first polar body

After the assessment of oocytes based on the expansion of cumulus cells, cumulus cells were removed by treatment of COCs with 0.1% Hyaluronidase in DPBS for three minutes and repeated pipetting through a fine glass pipette. Each oocyte was then observed under a stereo-zoom microscope to document the presence of the first polar body in the perivitelline space (Lv *et al.*, 2010; Nandi *et al*., 2004) [9, 10].

2.5 Statistical Analysis

The data obtained was analysed and compared using SAS 9.3 software.

3. Results and Discussion

The *in-vitro* maturation (IVM) performance of oocytes on the basis of cumulus cell expansion and extrusion of first polar body are presented in Table 1 and Table 2 respectively. The oocytes matured in goat follicular fluid group has shown significantly (p<0.05) higher per cent mean maturation rate (COCs expansion) and extrusion of first polar body (81.18±0.02), (58.02±0.02) than L-ascorbic acid group (72.21±0.02), (48.27±0.03) and Control (68.28±0.02), (38.90±0.03). L-ascorbic acid group has non-significant (p<0.05) in maturation rate than control. This significantly higher maturation rate of goat follicular fluid group found to be similar with Lakshmikanth *et al*. (2015) [8] and Wahjuningsih *et al*. (2014) [13] reported maturation rate of goat oocytes (78.50±2.4) and (85.23±7.52) respectively. However, the present study lower than Hoque *et al*. (2011) [6] reported (92.3±1.8) maturation rate based on COC expansion. The present study was found to be similar with Prasetyo *et al*. (2020) [11] and Almeeni *et al*. (2021) [2] reported maturation rate based on extrusion of first polar body was (61%) and (56%) in sheep oocytes.

Above results in goat follicular fluid group might be due to supplementation of goat follicular fluid [GFF] in a medium enhances the cumulus cell expansion and metaphase-II of the oocyte nucleus thereby increases the maturation rate because, follicular fluid contains protein, glucose, fatty acids as a source of nutrients, gonadotropin hormones, estrogen and mainly growth factors has an important role in the regulation of oocyte maturation via cumulus cells such as Epidermal Growth Factors [EGF], Transforming Growth Factors [TGF alpha], and Insulin Growth Factors-I [IGF-I] were important for the maturation of oocytes *in-vitro* (Wahjuningsih *et al.*, 2014) [13] and also the presence of intra-ovarian peptides in more physiological proportions in follicular fluid (Cognie *et al.*, 2004) [4].

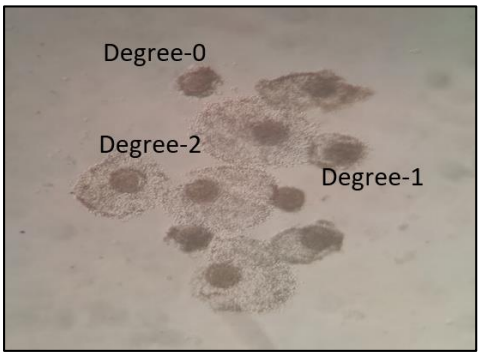

Fig 1: Assessment of COCs expansion

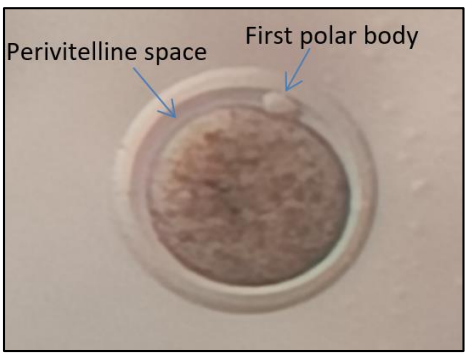

Fig 2: Extrusion of first polar body

Table 1: Maturation rate of goat oocytes in IVM Media supplemented with L-ascorbic acid and goat follicular fluid.

Maturation media	Number of COCs set for maturation	Degree of cumulus cell expansion						No. of matured oocytes		No. of polar body extruded	
		Degree-0	Percentage mean	Degree-1	Percentage mean	Degree-2	Percentage mean	(Degree-1 + Degree-2)	Percentage mean	No. of polar body observed	Percentage mean
Control (G-I)	112	35	31.72 ^{a}±0.02	40	35.63^{a}±0.02	37	32.65±0.02 ^{a}	77	68.28^{a}±0.02	45	38.90^{a}±0.03
L-ascorbic acid (G-II)	111	32	27.79 ^{a}±0.02	37	34.56^{a}±0.02	42	37.65±0.02 ^{a}	79	72.21^{a}±0.02	55	48.27$^{a\ b}$±0.03
Goat follicular fluid (G-III)	114	23	18.82^{b}±0.02	38	34.72 ^{a}±0.02	53	46.46 ^{b}±0.02	91	81.18^{b}±0.02	67	58.02^{b}±0.02

Means bearing different superscripts in the column differ significantly ($p \leq 0.05$).

Table 2: Percentage of maturation based on COCs expansion and polar body extrusion in different maturation media.

Maturation media	Based on Degree of cumulus cells expansion	Based on polar body extrusion
	Matured (Degree-1 + Degree-2)	% extrusion
Control (G-I)	68.28±0.02 ^{a}	38.90±0.03 ^{a}
L-ascorbic acid (G-II)	72.21±0.02 ^{a}	48.27±0.03 $^{a\ b}$
Goat follicular fluid (G-III)	81.18±0.02 ^{b}	58.02±0.02 ^{b}

Means bearing different superscripts in the column differ significantly ($p \leq 0.05$).

4. Conclusion

From the present experiment it can be concluded that, supplementation of goat follicular fluid in TCM-based medium has beneficial effect in improving *in-vitro* maturation rate of goat oocytes. Further studies are needed to validate the results and also to study the *in-vitro* fertilization rate.

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