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Effects of Time Specific Supplementation of Insulin Like Growth Factor-I (IGF-I) on *in vitro* Development of Sheep Preantral Follicles

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ABSTRACT

Background: In *in vitro* culture systems IGF-I was known to promote follicular maturation, granulosa cell proliferation and increase overall cellular function. The expression patterns of developmentally important markers for IGF-I are expressed differentially during the transition of preantral follicles to the ovulatory stage. Therefore, we aimed to investigate the influence of time specific addition of Insulin like growth factor-I (IGF-I) on *in vitro* development of sheep preantral follicles (PFs').

Methods: Mechanically isolated sheep preantral follicles were cultured for six days either in TCM 199B or standard medium supplemented with IGF-I at different points during the culture period and were subsequently subjected to *in vitro* maturation (IVM) for additional 24hrs to evaluate different follicular development parameters.

Conclusion: Based on the results, it is concluded that: 1) IGF-I supplementation was needed during early rather than later stages of culture period, therefore, role of IGF-I in time/stage specific manner in cultured preantral follicles is confirmed. 2) Supplementation of TCM 199B with IGF-I simultaneously for the first two days followed by TCM 199B alone without any growth factor (s) in later days (Treatment 1) supported better development of PFs' *in vitro.*

Key words: IGF-I, *In vitro* culture, Preantral follicles, Sheep.

INTRODUCTION

Several growth factors secreted locally in the ovarian follicle were shown to influence the growth, development and differentiation of both the somatic and germinal cells in the gonads of both the sexes in mammals (Canipari, 2000). A paracrine interaction between the developing oocytes and their surrounding follicular cells in response to growth factors is essential for the precise progression of both the follicles and oocytes to ovulation and subsequent development (Castro *et al.* 2015). Insulin like growth factor-I is one of such factor which plays an important role in the follicular maturation (Arunakumari *et al.* 2010), granulosa cell proliferation (Shiomi-Sugaya *et al.* 2015) and promotes normal ovarian activity (Magalhães *et al.* 2012). In *in vitro* culture systems IGF-I was known to stimulate DNA synthesis, steroidogenesis and increase overall cellular function (Rajarajan *et al.* 2006). Previous studies also revealed the expression patterns of several developmentally important genes for growth factors and hormones which were expressed differentially during the transition of preantral follicles to the ovulatory stage (Lakshminarayana *et al.* 2014; Praveen Chakravarthi *et al.* 2015; Kona *et al.* 2016). Thus it is likely that the use of hormones and growth factors needs to be modulated at different stages of development of the PFs' with an ultimate goal to develop an ideal culture system to obtain better *in vitro* growth of preantral follicles. Accordingly the present study was taken up with the objective of understanding the influence of time specific/sequential addition of Insulin like growth factor-I (IGF-I) and its interaction among different hormones on *in vitro* development of preantral follicles (PFs') in Sheep.

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MATERIALS AND METHODS

Unless otherwise stated, culture media, hormones, growth factors, FCS and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All the methods described briefly hereunder are routinely

employed in the culture of PFs' in the laboratory and described in detail in several earlier publication from the laboratory [Arunakumari *et al.* 2010; Kona *et al.* 2016; Anil kumar *et al.* 2019; Sravani Pragna *et al.* 2020].

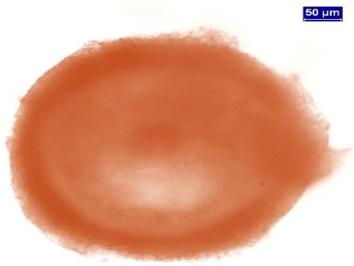
Isolation of preantral follicles [PFs']

The study was conducted at embryo biotechnology lab, S.V.V.U, Tirupati for which a total of 192 ovaries were collected from local slaughter house in Tirupati on different days during 2019-20 and 864 preantral follicles were isolated and cultured in nine different treatments. Ovaries recovered after sheep slaughter were transported to the laboratory within 1hr after slaughter in sterile, warm (37°C) phosphate buffered saline. Intact preantral (PFs') in the size range of 250-400µm with undamaged basement membrane were mechanically isolated (Fig 1A) by micro dissection method from ovarian cortex under a stereo-zoom microscope [SMZ 2T, Nikon corporation, Japan] and cultured them for six days (Fig 1B, 1C, 1D) according to the methods developed in our laboratory (Anil kumar *et al.* 2019; Sravani pragna *et al.* 2020). From each ovary 5-10 preantral follicles could be normally isolated.

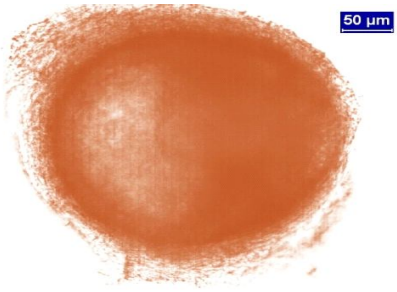
Culture media

To culture the sheep PFs' 10ng/ml Insulin like Growth factor-1 (IGF-I; I8779, Sigma, USA) was supplemented in time specific manner to base culture medium which includes either TCM 199B supplemented with 50µg/ml gentamycin

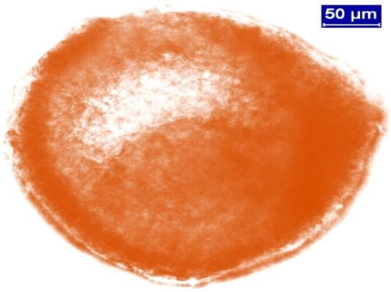
1A. Preantral follicle



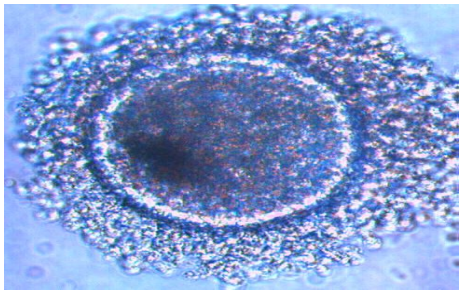
1B. Preantral follicle cultured for 2 days (20 X)



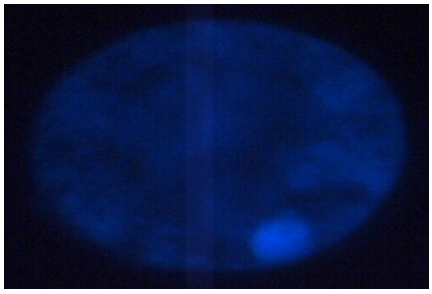
1C. Preantral follicle cultured for 4 days (20 X)



1D. Preantral follicle cultured for 6 days (20 X)



1E. Cumulus oocyte complex (COC)



1F. M-II oocyte

Fig 1: Development stages of the Sheep preantral follicles cultured *in vitro* during time specific supplementation of IGF-I.

(TCM199B) or Standard medium (SM; contains TCM199B supplemented with 50µg/ml gentamycin sulphate, 1 µg/ml Thyroxine (T_4), 2.5µg/ml Follicle stimulating hormone (FSH), 10ng/ml Insulin like Growth factor-1 (IGF-1) and 1 MIU/ml of growth hormone (GH). The experimental design indicating the different treatments of IGF-I supplementation during various culture durations was shown in (Table 1). All the culture media were pre incubated for 1hr at 39^{0}C under humidified atmosphere of 5% CO_2 in air.

Selection and culture of the preantral follicles

Intact preantral follicles in the size range of 250-400µm with intact basement membrane were selected for the culture (Fig 1A). The standard procedure was followed for culture of preantral follicles and *in vitro* maturation of oocytes from cultured PFs' which was explained in detail in previous studies in the laboratory [Arunakumari *et al.* 2010; Kamalamma *et al.* 2016; Anil kumar *et al.* 2019; Sravani Pragna *et al.* 2020]. The day on which the PFs' were placed in the culture was designated as day 0 and the subsequent days as day 1, 2 and so on. Half the medium was replaced by an equal volume of fresh medium every 48hrs and follicular development parameters under study were evaluated (Fig 1B, 1C, 1D). At the end of each experiment, cumulus oocyte complexes (COCs; Fig. 1E) were subjected to IVM to calculate the percentage of M II oocytes (Fig 1F).

Statistical analysis

The dependent variables were the development parameters of the follicles, and independent variables were the different treatments in the experiment. To analyse the effects of treatments on proportion of preantral follicles exhibiting growth, antrum formation, average increase in diameter and meiotic maturation of oocytes to M-II with respect to days of culture and their interactions two way ANOVA was employed. Duncan's multiple range test was applied to identify the significant differences. P values less than 0.05 were considered significant. SPSS 20 software was employed for all the above analyses.

RESULTS AND DISCUSSION

The influence of supplementation of IGF-I at different time points on development of sheep PFs' cultured *in vitro* was investigated for the first time in this study. It is interesting to note that IGF-I supplementation during first two days followed by TCM 199B for the rest of the culture (T 1, Table 2) significantly improved follicular development in terms of follicular growth, increase in diameter, antrum formation and meiotic maturation of oocytes to M-II compared to other treatments (Table 2). Since granulosa cells of follicles at initial stages during *in vitro* culture secrete IGF-I and its receptors (McCaffery *et al.* 2000, Walters *et al.* 2006; Jee *et al.* 2012; Magalhaes – Padilha *et al.* 2012 and Costa *et al.* 2014), present observations may be ascribed to the additive effects of externally supplied IGF-I along with indigenously secreted one. It is strike worthy that *in vitro* culturing of PFs' supplemented with IGF-I followed by TCM 199B resulted in better growth of the PFs' in terms of parameters studied than standard medium. TCM199B is a complex medium containing amino acids, vitamins, ribonucleosides, and deoxyribonucleosides in addition to the usual inorganic salts and energy sources (glucose). Structural rearrangements occur in the oocytes as the follicles develop towards graafian stage that aids in increased uptake of energy and nucleic acid synthesis which might be the crucial prerequisite in achieving meiotic developmental competence (Fair *et al.* 2003). Accordingly in our study, the base medium, TCM 199B

Table 1: Experimental design for time specific supplementation of Insulin like growth factor-I (IGF-I) for *in vitro* culture of Sheep preantral follicles [TCM 199B: Bicarbonate buffered Tissue culture medium 199; SM: Standard Medium]

Treatment	Acronyms used in this study	Details of the treatments
Treatment-T1	TI- IGF-I- 0-2;TCM 199B-3-6	Cultured in IGF-I for first 2 days (0-2) followed by culture in TCM 199B for 4 days (3-6 days)
Treatment-T2	T2-TCM 199B-0-2; IGF-I-3-4; TCM 199B-5-6	Cultured in TCM 199B for first 2 days (0-2)followed by culture in IGF-I for 2 days (3-4) days and then again cultured in TCM 199B for 2 days (5-6)
Treatment-T3	T3-TCM 199B-0-4; IGF-I-5-6	Cultured in TCM 199B for first 2 days (0-2) followed by culture in IGF-I for 4 days (3-6)
Treatment-T4	T4-IGF-I-0-6	Cultured in IGF-I for 6 days
Treatment-T5	T5-SM-0-6	Cultured in Standard medium (SM) for 6 days
Treatment-T6	T6-IGF-I-0-2; SM-3-6	Cultured in IGF-I for first 2 days (0-2) followed by culture in Standard medium for 4 days (3-6 days)
Treatment-T7	T7-SM-0-2; IGF-I-3-4; SM-5-6	Cultured in Standard medium for first 2 days (0-2) followed by culture in IGF-I for 2 days (3-4) days and then again cultured in Standard medium for 2 days (5-6)
Treatment-T8	T8-SM-0-4; IGF-I-5-6	Cultured in Standard medium for first 2 days (0-2) followed by culture in IGF-I for 4 days (3-6)
Treatment-T9	T9-TCM 199B-0-6	Cultured in TCM 199B for 6 days

Table 2: Influence of supplementation of Insulin like growth factor (10 ng/ml) at different time points during six day *in vitro* culture of sheep preantral follicles.

Treatments (16/96)	Proportion (%) of PFs' Exhibiting Growth (Mean ± SE)	Average Increase in Diameter (µm) of PFs' (Mean ± SE)	Proportion (%) of PFs' Exhibiting Antrum Formation (Mean ± SE)	Proportion (%) of oocytes matured to M-II* (Mean ± SE)
TI-IGF-I-0-2;TCM199B 3-6	98.89±1.67^{a}	42.50±1.73^{a}	96.87±1.67^{a}	23.00±4.09^{a}
T2-TCM199B-0-2; IGF-I-3-4; TCM199B-5-6	85.46±1.52^{b}	24.88±0.80^{b}	79.16±3.56 ^{b}	13.00±0.61^{b}
T3-TCM199B-0-4;IGF-I-5-6	82.18±3.93^{b}	21.81±0.91^{b}	80.2±4.36 ^{bc}	15.00±0.86^{b}
T4-IGF-I-0-6	86.11±6.72^{b}	28.88±1.84^{b}	84.37±8.25^{bc}	13.00±0.61^{b}
T5-SM- 0-6	84.79±5.68^{b}	23.19±0.65^{b}	72.91±6.06^{b}	10.00±3.90^{b}
T6- IGF-I-0-2; SM-3-6	86.67±3.60^{b}	33.13±1.76^{c}	78.12±4.22^{b}	19.00±2.83^{b}
T7-SM-0-2; IGF-I-3-4; SM-5-6	80.99±0.90^{b}	24.06±1.57^{b}	82.28±3.55^{bc}	9.00±5.52^{b}
T8-SM-0-4; IGF-I-5-6	86.78±3.86^{b}	24.88±1.48^{b}	85.41±3.68^{bc}	11.00±2.58^{b}
T9-TCM199B-0-6 (control)	78.16±7.43^{b}	22.62±0.59^{b}	77.49±3.87^{b}	11.00±2.63^{b}

Figures with different superscripts within a column are significantly different ($P \leq 0.05$).

* oocytes in COCs isolated from six day cultured follicles in different treatments and subjected to IVM for additional 24hrs

SM = Standard Medium.

was able to restrict the need for growth factors during initial period of culture. Although it was reported that inclusion of IGF-I for the entire length of culture resulted in better proportion of M-II oocytes (Zhou and Zhang 2005; Arunakumari *et al.* 2010,; Magalhães *et al.* 2012; Luz *et al.* 2013; Teja *et al.* 2013; Costa *et al.* 2014; Jimenez *et al.* 2018), in this study culturing of PFs' in medium supplemented with IGF-I for first two days (T1; Table 2) showed increased proportion of oocytes reaching Metaphase-II which could be likely the direct action of IGF-I on its receptors in the oocytes and granulosa cells of follicles in the early stages of development (Walters *et al.* 2006; Jee *et al.* 2012; Magalhaes – Padilha *et al.* 2012 and Costa *et al.* 2014) or it is also possible that the effects of the presence of IGF-I during early culture period might have been carried forward into later stages of development. It is remarkable that the treatment which had increased the diameter of PFs' in culture (T1) was the one which supported higher meiotic maturation of oocytes subsequently (Table 2). There were reports supporting this observation which indicated that higher diameter is a prerequisite for meiotic maturation of oocytes (Grasselli *et al.* 2002; Bruno *et al.* 2009 and Bui *et al.* 2016), therefore, the better meiotic maturation of oocytes in T1 could be related to the better diameter achieved by the follicles. Since time specific inclusion of IGF-I for initial two days (T1) supported the PFs' in achieving better follicular diameter during culture and since increase in diameter is invariably a result of increase in the number of follicular cells and oocyte size, it is suggested that IGF-I influenced the PFs' in the early days of culture through its mitogenic activity in granulosa cells and cumulus cells (Guthrie *et al.* 1998; Louhio *et al.* 2000; Demeestere *et al.* 2004). As no studies were conducted on the mechanism of action of IGF-I in the development of PFs' in this study, it is assumed that role of IGF-I in DNA synthesis (Rajarajan *et al.* 2006), granulosa cell proliferation (Shiomi-Sugaya *et al.* 2015), mitosis in cumulus cells (Zhao *et al.* 2001), stimulation of gap junctional based communication (Demeestere *et al.* 2004) or anti apoptotic action on granulosa cells (Mao *et al.* 2004) might be responsible for the beneficial effects of IGF-I in the culture. In our study we observed the time specific role of IGF-I in cultured sheep PFs' and we conclude that IGF-I supplementation was needed during early phase of culture *i.e.* for the first two days followed by TCM 199B alone without any growth factor(s) in later days (T1; Table 2) which supported better development of PFs' *in vitro.*

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Conflict of interest

Authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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