Full Length Article

EFFECT OF SUPPLEMENTATION OF ESTRADIOL AND GROWTH HORMONE ON *IN VITRO* DEVELOPMENT OF PREANTRAL FOLLICLES IN SHEEP

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ABSTRACT

The aim of this study was to investigate the effects of addition of estradiol and growth hormone on in vitro growth, maturation and antrum formation of preantral follicles (PFs') in sheep. Preantral follicles isolated from the sheep ovarian cortical slices were cultured for six days in bicarbonate buffered tissue culture medium 199B, standard culture medium supplemented with estradiol and growth hormone during the culture period at different time points. Cumulus oocyte complexes (COCs) segregated from the follicles toward the end of six days culture in various treatments were exposed to in vitro maturation for extra 24hrs. Estradiol plus growth hormone supplementation during 0-2 days of the culture significantly ($P \leq 0.05$) increased the average diameter of PFs' and supported better for antrum formation. Further the in vitro maturation (IVM) of oocytes in COCs isolated were developed to metaphase II stage at a higher rate. First two (0-2) days supplementation with estradiol plus growth hormone to TCM 199B in vitro culture medium followed by standard medium in later stages (3-6 days) supports better development of PFs' and appears to be advantageous for the development of oocytes to M-II stage.

Keywords: Estradiol, Growth hormone, *In vitro* maturation, Preantral follicles, Sheep

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INTRODUCTION

The reserve primordial follicles in the ovary of mammalian females are abundant. However, a small portion grows to become graafian follicles and reaches the stage of ovulation, but the remaining primordial follicles become atretic during their growth or maturation (Reddy *et al.,* 2021). The development of culture systems to promote the growth and maturation of preantral follicles (PFs') up to the graafian follicle stage would be useful to avoid this degeneration

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of follicles due to atresia. This fact has created great interest in the development of *in vitro* culture system of ovarian follicles to maintain follicular growth and avoid follicular degeneration (Araújo *et al.,* 2014). The *in vitro* follicle culture has succeeded in laboratory animals like mice, where birth of live offspring was obtained after fertilization of oocytes from *in vitro* cultured preantral follicles (PFs'). On the other hand, in large animals like sheep, such an achievement has not been reported. Instead, the results have been limited to the production of a low and variable number of metaphase II (M-II) oocytes and embryos. Those results were obtained using culture medium supplemented with several components like gonadotropins, growth hormone, estradiol and growth factors etc. Several hormones and growth factors' influence the *in vitro* development of sheep PFs'. (Pragna *et al.,* 2020; Reddy *et al.,* 2021). However, the most promising results are obtained by adding these growth factors and hormones to the culture medium in different time points. Stage-specific supplementation of growth factors and hormones are necessary during *in vitro* culturing of PFs' for better follicular development, follicle health and ovarian tissue viability (Peng *et al.,* 2010).

Some *in vitro* studies have demonstrated that the estradiol can act as apoptosis inhibitor in the follicular cells (Lima-Verde *et al.,* 2010; Murdoch, 1998). Granulosa cells become receptive to gonadotrophin and growth hormone in the presence of estradiol, which may act to support granulosa cell differentiation. Estradiol promotes cell proliferation and survival by activating the transcription of factors required for cell cycle progression, while inhibiting other factors that cause cell cycle arrest and apoptosis (Craig *et al.,* 2014). Some *in vitro* studies suggested that GH plays a role in follicular growth during gonadotropin-independent stages of folliculogenesis and could have a direct inhibitory action on follicle apoptosis. GH promotes PFs' development *in vitro* and supports the morphology of cultured PFs' (Zhao *et al.,* 2000a).

However, there are no reports showing the interaction between estradiol and growth hormone in promoting the growth of preantral follicles in sheep at different time points. Therefore, the present study was undertaken to ascertain the influence of estradiol plus growth hormone on *in vitro* development of preantral follicles (PFs') in sheep at different time points.

MATERIALS AND METHODS

Sheep ovaries (n=20) collected from a local slaughter were washed with warm PBS immediately after collection. The ovaries were kept in polythene sachets containing warm (37°C) PBS and transported to the laboratory within one hour after collection in a thermos flask containing warm water (37°C). On reaching the laboratory, working area was cleaned with 70 percent alcohol and the ovaries were handled aseptically. The ovarian cortex was cut into thin slices from which intact preantral follicles in the size range of 250 - 400 µm were mechanically isolated by micro-dissection method under stereo zoom microscope.

The basic control medium utilized in the study was tissue culture medium 199

(TCM 199B) and standard medium for *in vitro* follicular culture was prepared as per the procedure described by Arunakumari *et al.* (2010). The composition of standard medium was as follows:

1. TCM 199B - 9275µl/ml
2. IGF-1 - 200 µl/ml
3. GH - 200 µl/ml
4. FSH - 175µl/ml
5. T_4 - 100µl/ml
6. Gentamycin - 50 µl/ml

Treatment groups are divided into a total of nine (T1 to T9) as represented in Table 1. Preantral follicles were cultured in micro droplets of TCM 199B / standard medium for six days, supplemented with estradiol (5 ng/ml) and growth hormone (1 mIU/ml). The morphological evaluation of the cultured follicles was performed every 24h to assess the proportion of growth, the average increase in diameter and proportion of PFs' exhibiting antrum formation. At the end of six-day culture, the cumulus oocyte complexes (COCs) were harvested and matured *in vitro* for an additional 24 hours. Meiotic maturation to stage M-II was assessed by staining the oocytes with Hoechst 33342 medium.

The data of the study were analyzed by one way ANOVA (SPSS 20) followed by Duncan's multiple range test (DMRT) and percentile deviation tests to determine the significance of difference among various treatment groups.

Table 1. Experimental design for time specific supplementation of estradiol (5 ng/ml) and GH (1 mIU/ml) on *in vitro* culture of preantral follicles in sheep

Treatment	Details of the treatments
Treatment 1 (T1)	Cultured 0-6 days in TCM 199B
Treatment 2 (T2)	Cultured 0-6 days in Standard medium (SM)
Treatment 3 (T3)	Cultured 0-6 days in Estradiol + GH
Treatment 4 (T4)	Cultured 0-2 days in Estradiol + GH, 3-6 days in TCM 199B
Treatment 5 (T5)	Cultured 0-2 days in Estradiol + GH, 3-6 days in Standard medium
Treatment 6 (T6)	Cultured 0-4 days in TCM 199B, 5-6 days in Estradiol + GH
Treatment 7 (T7)	Cultured 0-4 days in Standard medium, 5-6 days in Estradiol + GH
Treatment 8 (T8)	Cultured 0-2 days in TCM 199B, 3-4 days in Estradiol + GH and 5-6 days again in TCM 199B
Treatment 9 (T9)	Cultured 0-2 days in Standard medium, 3-4 days in Estradiol + GH and 5-6 days again in Standard medium

RESULTS AND DISCUSSION

This study ascertained the effect of estradiol and growth hormone on *in vitro* development of sheep preantral follicles at different time points. Estradiol plus growth hormone supplementation during 0-2 days of the culture and followed by 3-6 days standard medium supplementation (T5 group) significantly ($P \leq 0.05$) increased the average diameter of PFs' and better supported the growth rate of PFs' (Table 2). Antrum formation and oocyte maturation rate was also increased in T5 group (Fig. 1 & 2). This study showed the importance of estradiol and growth hormone in growth, activation and maturation of sheep preantral follicles.

In the present study the PFs' exhibited higher proportion of growth, diameter, antrum formation and *in vitro* maturation of oocytes to M-II stage when PFs' were cultured in the medium supplemented with estradiol (5 ng/ml) plus growth hormone (1 mIU/ml). This was in agreement with Reddy *et al.* (2021), who reported that the estradiol with LH supplementation at 0-2days to *in vitro* culture followed by standard medium for 3-6 days supports better growth and antrum formation in PF's of sheep. Whereas, Magalhães -Padilha *et al.* (2012) and Hull and Harvey (2001), reported that the GH stimulates growth and prevents atresia to maintain survival of ovarian follicles. The GH at lower concentrations (1ng/ml bGH) increased survival rates in rats PFs' (Zhao *et al.,* 2000b) and increased the DNA content and growth rate in the cultured rat PFs' (Liu *et al.,* 1998).

In vitro studies have shown that GH has a direct inhibitory effect on apoptosis in early bovine (Sirotkin and Makarevich, 1999) and rat (Eisenhauer *et al.,* 1995) follicles. The mechanism by which GH regulates survival

Table 2. Influence of supplementation of estradiol (5 ng/ml) and GH (1 mIU/ml) at different time points during *in vitro* culture of sheep preantral follicles (PFs')

Treatments	Proportion (%) of PFs' Exhibiting Growth (Mean ± SE)	Average Increase in Diameter (µm) of PFs' (Mean ± SE)
T1	63.33 ± 3.33^{a}	18.00 ± 2.86^{b}
T2	66.66 ± 3.51^{a}	21.33 ± 2.86^{ab}
T3	73.33 ± 5.66^{a}	27.66 ± 1.72^{ab}
T4	70.00 ± 4.15^{a}	23.66 ± 2.70^{ab}
T5	76.66 ± 6.18^{a}	30.83 ± 1.70^{a}
T6	66.66 ± 3.51^{a}	23.50 ± 2.05^{ab}
T7	68.33 ± 5.24^{a}	28.99 ± 1.86^{a}
T8	68.33 ± 3.88^{a}	23.16 ± 3.53^{ab}
T9	71.66 ± 4.99^{a}	29.16 ± 1.79^{a}

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

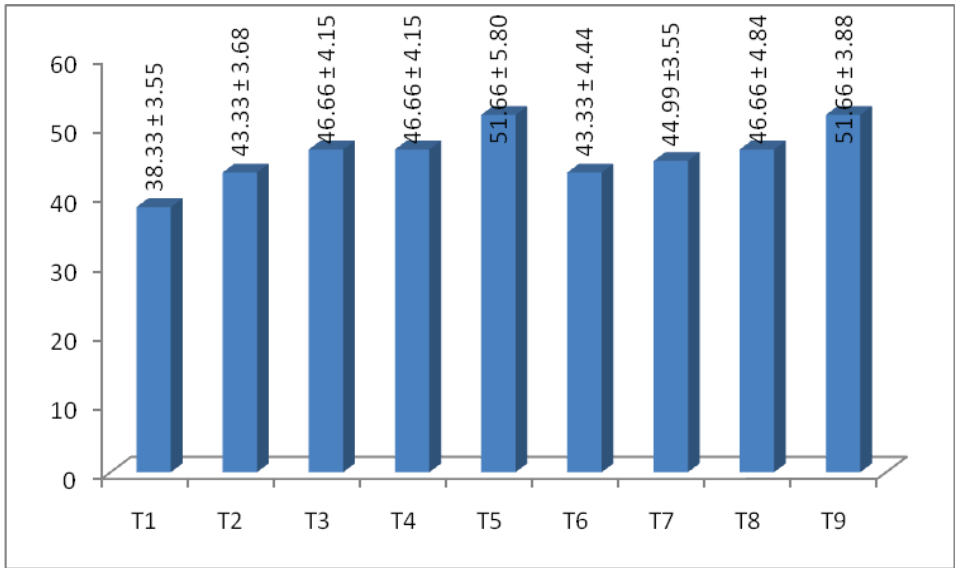

Fig. 1. Proportion (%) of PFs' exhibiting antrum formation (Mean ± SE)

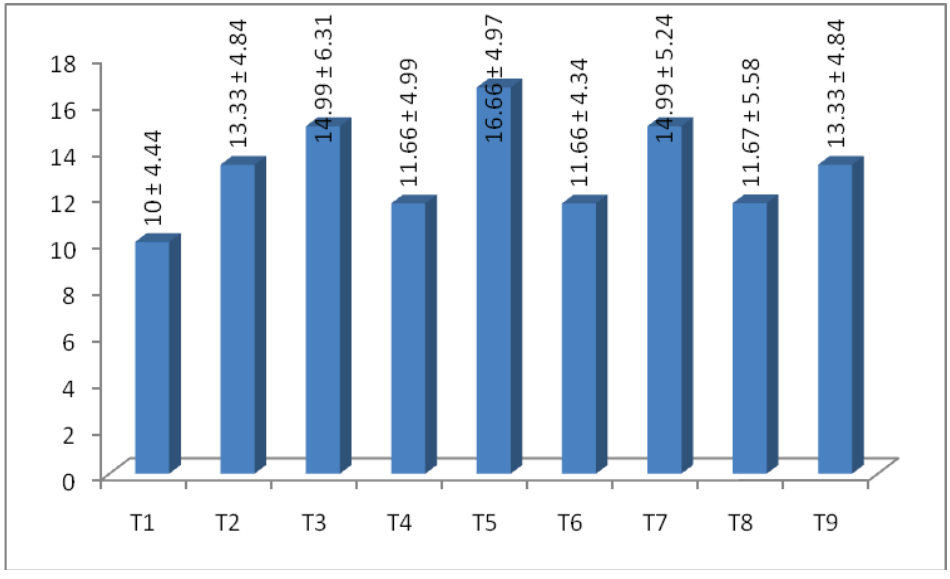

Fig. 2. Proportion (%) of oocytes matured to M-II* (Mean ± SE)

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

in ovarian follicles is not exactly known yet. The local action of GH could be through an autocrine or paracrine mechanism, although the endocrine mechanisms of action of GH have been more reported (Magalhães-Padilha *et al.,* 2012). GH is able to accelerate the cytoplasmic maturation of the PFs and the role of GH in follicular growth may be associated with its reported effects on cellular proliferation and steroidogenesis, considering that steroid hormones secreted from COCs are essential for meiotic maturation and cumulus expansion (Magalhaes *et al.,* 2011). The evidence indicates that growth hormone (GH), in addition to its metabolic effects, is involved in the regulation of ovarian folliculogenesis.

The results of our study, revealed that the supplementation of estradiol and growth hormone has significantly ($P \leq 0.05$) increased the average diameter of PFs'. This could be due to inhibition of apoptosis of luteal and follicular cells by supplemented estradiol (Murdoch, 1998). Our results are in accordance with the findings of Tasaki *et al.* (2013), who observed that the pig PFs' cultured in medium containing 1 µg/ml estradiol exhibited highest rate of antrum formation and PFs' cultured in medium without estradiol exhibited no antrum formation. Lima-Verde *et al.* (2010) observed that the supplementation of estradiol promoted follicular growth and activation of *in vitro* caprine primordial follicles. Similar results were reported by Endo *et al.* (2013), who observed that the estradiol supplementation to the culture medium improved the cavity formation, and the highest antrum cavity formation ratio. Proper time and levels of estradiol promotes follicle growth and inhibit follicle atresia.

In our study initial supplementation (0-2days) of estradiol and GH showed the higher proportion of PFs' exhibited growth and increase in diameter, although antrum formation and *in vitro* maturation of oocytes to M-II stage were not significantly increased. It clearly suggests that effect of estradiol and GH in early stage have been effective on *in vitro* culture of PFs' in sheep. Therefore, we inferred that estradiol along with GH act as a regulator of follicular development during the initial phase of *in vitro* culture.

CONCLUSION

It was concluded that the supplementation of estradiol and GH to TCM 199B culture medium in the first two days (0-2 days) and the standard medium alone in later period (3-6 days) resulted in better average increase in diameter and proportion of PFs' that exhibits antrum formation at the end of six day culture. In addition, the oocytes in COCs isolated at the end of the culture and subjected to 24hours of *in vitro* maturation (IVM) to develop at a higher frequency until the M-II (metaphase II) stage.

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