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In vitro maturation of sheep prepubertal oocytes

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Abstract
The aim of the study was to determine concentration of the Estrus sheep serum (ESS) for sheep *in vitro* oocyte maturation. Sheep ovaries were collected from a local abattoir and transported within 1 h to the laboratory in a warm saline solution (30 – 35 ºC), Cumulus-oocyte complexes (COC's) were obtained by aspirating of follicles, washed in TCM-199 modification with 50 g/ml gentamycin, The COC's were randomly divided into Three groups. Treatment I (n = 100) COC's were fresh and cultured in TCM-199 medium with 10% ESS + Estrdio 17β + 10 µg/ml FSH+10µg/ml LH. Treatment II (n = 100) COC's were washed five times and cultured in TCM-199 medium supplemented with Estrdio 17β + 10 µg/ml FSH+10µg/ml LH. Treatment III (n = 100) COC's were washed five times and cultured in TCM-199 medium and gentamycin treated as control. The evidence of different stages of maturation. Significantly higher (p<0.05) maturation rates of oocytes (95%, 61% and 10% were observed in Treatment I, IIand III However, significant difference was observed between the Treatment I,II and III.

Keywords: Shepp prepubertal, Estrus sheep serum, Oocyte maturation: Ovary

Introduction
Sheep is an important species of livestock around the world and especially in India contributing greatly to mutton and wool in rural development. India possesses total sheep population of 65.06 millions, which contribute wool 47.9 million kg (FAO 2015-2016) and 65.06 million tonnes of meat (FAO 2015-2016). Improvement of reproductive efficiency of sheep through technologies such as artificial insemination, embryo transfer and cloning could enhance their contribution to the Indian economy. Sheep (*Ovisaries*) can be used as the best farm animal for reproductive research because of their earlier age of puberty, short gestational periods, the twinning character and the lower maintenance cost. Moreover, sheep have been domesticated for so many years and are also widespread across the world, being adapted to many different climatic conditions. The incorporation of juvenile *in vitro* embryo technology into breeding programmes is advantageous because it can reduce the generation interval and increase the genetic gain

Material Methods
Handling medium for oocytes: TCM 199 H was supplemented with 10% Estrus Sheep Serum, 25 IU/ml of heparin (H3393, Sigma, USA) and 50µg/ml gentamicin sulphate. The medium was sterilized by filtration through a0.22µm filter and incubated at 39 ºC under humidified atmosphere in 5% CO_2 in air for 1 h prior to use

Treatment I
TCM 199 B was supplemented with 10µg/ml FH, 10µg/ml Luteinizing hormone (L9773,Sigma,USA), 1µg/ml estradiol -17β(E4389,Sigma, USA), 50µg/ml gentamicin sulphate, 10% (v/v) estrus sheep serum (ESS).This was stored at 4 ^{0}C for up to 1 week in a 5ml disposable syringe with 0.22µm filter attached. Filtered medium was equilibrated for 1h at 39 ^{0}C in 5% CO_2 in air under humidified atmosphere prior to use.

Treatment II
TCM 199 B was supplemented with 10µg/ml FH, 10µg/ml Luteinizing hormone (L9773, Sigma, USA), 1µg/ml estradiol -17β (E4389,Sigma, USA), 50µg/ml gentamicin sulphate This was stored at 4 ^{0}C for up to 1 week in a 5ml disposable syringe with 0.22µm filter attached. Filtered medium was equilibrated for 1h at 39 ^{0}C in 5% CO_2 in air under humidified atmosphere prior to use.

Treatment III
TCM 199 B was supplemented with 50µg/ml gentamicin sulphate. Filtered medium was equilibrated for 1h at 39 ^{0}C in 5% CO_2 in air under humidified atmosphere prior to use.

Collection of estrus sheep serum (ESS)
Blood was collected aseptically from the jugular vein of ewes on the day of standing heat into sterile glass test tubes. The blood was allowed to clot for 1to 2h.After the clot formation, the test tubes were transferred to a refrigerator (4-5 ºC) and serum was allowed to ooze. Then the serum was separated and centrifuged at 1000rpm for 10 min. The separated serum was filtered through a 0.22µm filter. The complement in serum was inactivated by treating at 56 ºC for 30 min in hot water bath. Then it was stored in 1 ml aliquots in 2ml lymphocyte tubes (349638, Nalge, Nunk, Denmark) at 20 ºC till used.

Collection and processing of ovaries
Ovaries from sheep were recovered from local slaughter house at Ziaguda, Hyderabad, immediately after slaughter. The ovaries were washed with warm PBS and kept in polythene sachets containing warm (37 ºC) PBS. These sachets were transported to the laboratory within 5-6 h after collection in a thermos flask containing warm water (37 ºC). On reaching the laboratory, working area was cleaned with 70% alcohol. The tissues and ligaments were trimmed and the ovaries were washed twice in PBS supplemented with 50µg/ml gentamicin sulphate. Then they were immersed in 70% alcohol for 3-5sec and rinsed twice in PBS with antibiotics. All the subsequent procedures were carried out in a laminar air flow (Nuair, Class II type A B3, USA).

***In vitro* Maturation of oocytes from *in vitro* cultured follicles**
The oocytes collected from *in vitro* cultured follicles were washed thrice in the *in vitro* maturation medium. Subsequently, oocytes were placed individually in 20µl droplets of IVM mediumin 35mm tissue culture dishes. The droplets were overlaid with autoclaved pre-equilibrated light weight mineral oil. These culture dishes were incubated at 39 ºC in 5% CO_2under humidified atmosphere for 24 h.

Evaluation of IVM oocytes
At the end of IVM period, the oocytes were denuded off cumulus cells by repeated pipetting through a fine bore glass pipette. Subsequently the oocytes were stained with propidium iodide and incubated in a 50µl droplet of incubation period for 30 minutes. Then the oocytes were examined under fluorescent light on an inverted microscope and recorded the maturation rate.

Statistical Analysis
Data on COC (cumulus-oocyte complexes) expansion and extrusion of Polar body were analyzed using SPSS Version 17. Mean and standard error were calculated for each group and were compared by one-way analysis of variance (ANOVA). Duncan's test was used for the multiple comparisons of values for calculation of significant difference between control group and treatment groups. Differences were considered significant when$p \leq 0.05$.

Results and Discussion
In treatment I,TCM 199B supplemented with hormonesFSH10µg/ml,LH10µg/ml and 10% estrus sheep serum (ESS) to identify the best to support oocytes maturation in terms of cumulus cell expansion (CCE) and extrusion of 1st polar body (PB).Among total of 300 culture grade oocytes (replicates: 5), CCE was found highest in treatment I (95±0.00), followed by II(61 ±0.86), while it was lowest in the control (III; 10±0.54).There was significant (P<0.05) difference among the groups.
All the three supplemented with TCM 199B maturation media supported to extrude PB. Among three treatments (I, IIand III), highest proportion (57±0.24) of oocytes extruded PB when COCS were cultured in the medium supplemented with TCM 199B and hormones with estrus sheep serum and the lowest proportion was observed in control(gentamicin; 4±0.37).Among the three treated groups, the proportion of oocytes extruded PB with ESS was significantly (P<0.05) higher than IVM media, hormones (41±0.37) and control(4±0.37).Among three treatments (I, IIand III), highest proportion (51±0.73.) of oocytes germinal vesicle were observed in the medium supplemented with TCM 199B and hormones with estrus sheep serum and the lowest proportion was observed in control (8±0.24). Among the TCM 199B treated groups, the proportion of oocytesgerminal vesicle break down in treatment I (48±0.50) was significantly (P<0.05) higher than II(31±0.80) and control (6±0.20). Among three treatments (I, IIand III), highest proportion (48±0.50.) of oocytes germinal vesicle break down was found in the medium supplemented with TCM 199B and hormones with estrus sheep serum and the lowest proportion was observed in control gentamicin (6±0.20). Among the three treated groups, the proportion of oocytesgerminal vesicle in TCM 199B ESS (I) was significantly (P<0.05) higher than TCM 199B hormones (II; 48±0.50) and control (III; 6±0.20) Table 1

TABLE 1: Maturation Rate of Prepubertal Sheep Oocytes Using Different Ivm Media

Maturation Medium	No of oocytes/ Replicates	Cumulus cell expansion	Extrusion of 1st polar body	G.V	G.V.B.D
Treatment I (TCM 199B+10% ESS+FSH+10 µg/ml+ LH 10µg/ml+Estradiol 17 β1 µg/ml)	100/5	95±0.00^a	57±0.24^a	51±0.73^a	48±0.50^a
Treatment II (TCM 199B+FSH+10 µg/ml+ LH 10µg/ml+Estradiol 17 β1 µg/ml)	100/5	61±0.86^b	41±0.37^b	37±0.60^b	31±0.80^b
Control (TCM 199B + gentamicin	100/5	10±0.54^c	4±0.37^c	8±0.24^c	6±0.20^c

Means with different alphabets (a, b and c) as superscripts within column differ significantly (P<0.05)

The culturable oocytes were matured for 24 h in maturation media (TCM 199H supplemented with 10% estrus sheep serum (ESS), FSH10µg/ml and LH 10µg/mlyielded95±0.00% of cumulus cell expansion, which was similar to the report of Sharma *et al*. (2015) [3, 8] in goats, Mishra *et al*. (2008) [3] in buffaloes and Priyanka *et al*. (2017) [5] in sheep. Further, observation of Extrusion of polar body was 57±0.24%, which was similar to the findings of the observations of germinal vesicle in Treatment I was51±0.73%, which was significantly higher when compared to other treatments. These results are

in accordance with the reports of Shirirazi *et al*. (2006) [9]. On the contrary, Shankariah *et al.* (2013) [7] and Divya (2014) reported lesser values. This variation observed in maturation rate could be due to many reasons asmaturation rate is influenced by several factors like presence of follicular cells, protein supplementation, hormones, antioxidants and growth factors in maturationmedia (Kharche *et al*., 2011) [1]. The reasons for goodmaturation rate in our experiment may be due to 10% ESS in addition to IV Mmedium, FSH (10 µg/ml) and LH(10 µg/ml)). Mogas *et al*. (1997) [4] reported that addition of FSH, LH and estradiol increased the maturation rate in caprine oocytes. Similarly, reported that addition of estrus sheep serum could enhance the *in vitro* maturation and development of *in vitro* matured oocytes. The percentage of germinal vesicle breakdown in Treatment I was 48±0.50, which was higher when compared to other treatments. The findings of this study are in accordance with the results of Shabankareh *et al.* (2011) [6], Ledda *et al.* (1999) [2] and Lei Shi *et al*. (2009) [10].

Conclusion

In vitro maturation rate of prepubertal sheep oocytes by using different IVM media revealed that the treatment I was found superior to the remaining treatments.

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