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# Characterization of Leptin receptor protein (LepR) expression in sheep ovarian follicles grown *in vivo* and cultured *in vitro* in different media

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**Abstract**
Leptin receptor (LepR) protein expression in sheep was studied through Immunohistochemistry from: (i) *In vivo* grown preantral (PFs'), early antral, antral, large antral follicles and COCs obtained from large antral follicles subjected to 24hrs of *in vitro* maturation and (ii) Preantral follicles exposed to TCM199B, TCM199B+Leptin and standard medium+Leptin for 3min, two, four or six days and subsequently matured *in vitro* for 24hrs. LepR immunolocalization was observed at all stages of ovarian follicles with highest intensity of protein expression in the oocytes but mild to moderate expression was observed in cumulus cells. From our study it is concluded that culture of preantral follicles in medium containing Leptin concomitantly supplemented with other growth factors and hormones stimulated the protein expression better than in other groups therefore indicating the additive effects of Leptin with other growth factors and hormones in promoting the preantral follicular development towards ovulatory phase.

**Keywords:** immunohistochemistry, leptin receptor protein, preantral follicles, sheep

## 1. Introduction
Leptin, a transmembrane receptor protein has beneficial effects on the follicular development, oocyte maturation (Kamalamma *et al.*, 2016) [11] and therefore involved in the regulation of female reproductive system through its actions on the gonadotrophic axis and ovaries (Catteau *et al.*, 2015). In our laboratory the optimum dose of leptin that supports better development of preantral follicles during culture was standardized at 10 ng/mL (Kamalamma *et al.*, 2016) [11]. Previous studies indicated that leptin was able to modulate its receptor expression in differential manner depending on the dose and duration of hormone supplementation (Ricci *et al.*, 2006; Di Yorio *et al.*, 2008; Cordova *et al.*, 2011) [15, 7, 6]. It is not exactly known whether the role of leptin in promoting the preantral follicle development in sheep is through its regulation of own receptor activity in ovaries. Also based on the LepR activity at different stages of preantral follicular development it will be possible to design better medium involving various growth promoters including leptin at specific stages of follicle development during culture which will further enhance the overall development of preantral follicles in culture conditions and therefore the better meiotic maturation of oocytes. In this regard to identify the effects of leptin supplementation on its receptor protein expression the present study was undertaken.

## 2. Materials and Methods
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; Kamalamma *et al.*, 2016; Kona *et al.*, 2016; Anil kumar *et al.*, 2019; Sravani pragna *et al.*, 2020] [3, 11, 12, 2, 18]. 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 hormones and growth factors used were cell culture tested and endotoxin free.

### 2.1 Collection, processing and Isolation of preantral follicles [PFs'] from sheep ovaries
Ovaries (n=127) recovered immediately after sheep slaughter on different days were transported to the laboratory within 1hr after slaughter in sterile, warm [37 $^0$C] phosphate

buffered saline. A total of 640 intact preantral [PFs'] in the size range of 250-400µm with undamaged basement membrane were mechanically isolated [Figure 1: A] by micro dissection method from ovarian cortex under a stereo-zoom microscope [SMZ 2T, Niko n corporation, Japan] and cultured them for six days in different groups with subsequent *in vitro* maturation [Figure 1: B-D] according to the methods developed in our laboratory [Arunakumari *et al.,* 2010; Kona *et al.,* 2016] [3, 12]. *In vivo* developed follicular stages (Group I) including intact preantral (PFs'), early antral, antral, large antral follicles were mechanically isolated by micro dissection method from ovarian cortex under a stereo-zoom microscope (SMZ 2T, Nikon corporation, Japan) and also cumulus oocyte complexes (COCs) were aspirated from graafian follicles [Figure 1: E] according to the methods developed in our laboratory (Arunakumari *et al.,* 2010; Kona *et al.,* 2016) [3, 12]. PFs' in the size range of 250 – 400 µm having visible centrally placed oocytes with no signs of atresia and with intact basement membrane and no antral cavity were considered as good quality follicles and were used for culture.

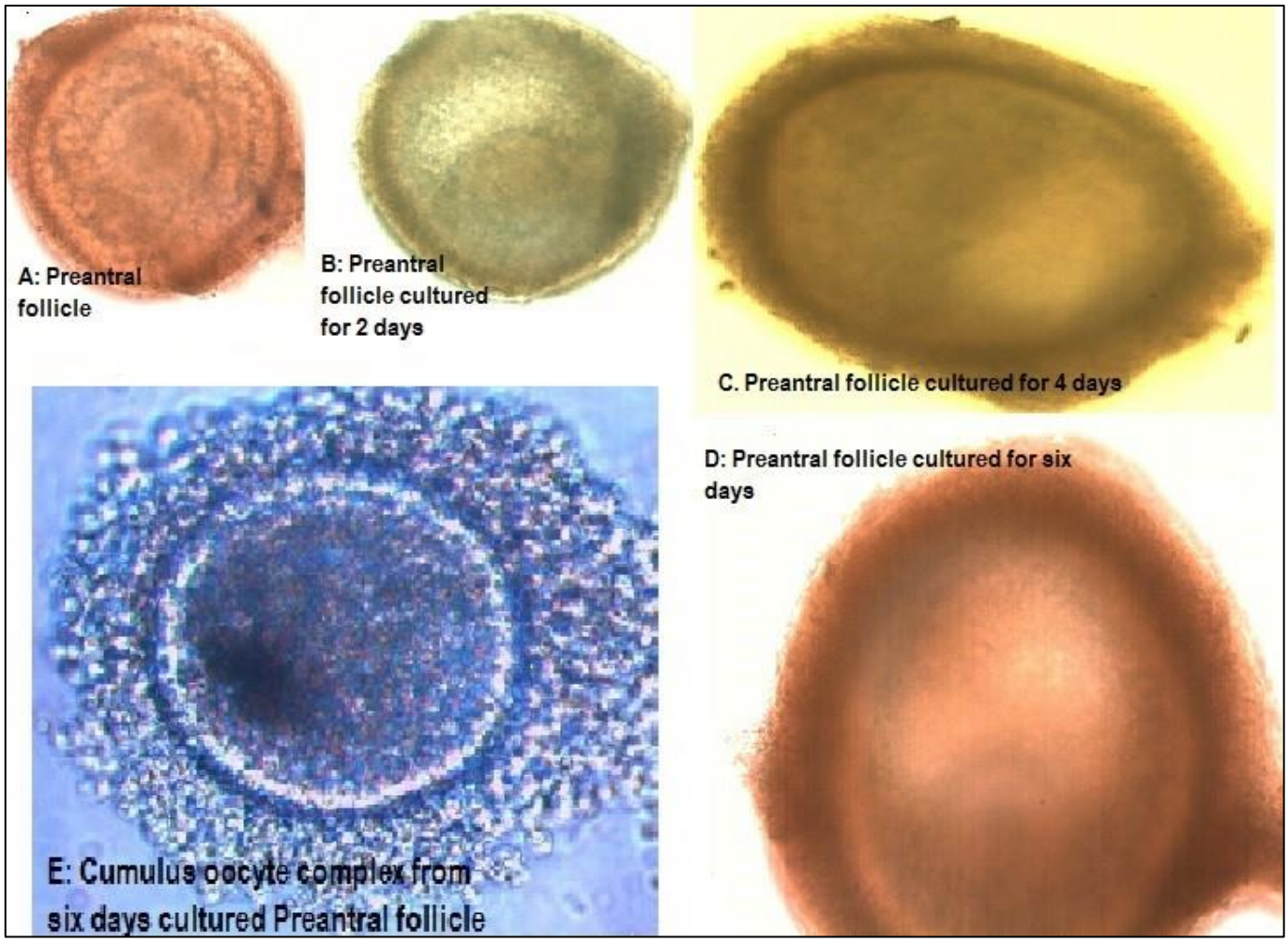


**Fig 1(A-E)**: Different stages of the Sheep preantral follicles during 3 min, 2 days, 4 days and 6 days of *in vitro* culture and subsequent *in vitro* matured cumulus oocytes complex during supplementation of Leptin to culture media**.**

**2.2 Media preparation and culture of preantral follicles**

Preantral follicles were cultured in bicarbonate buffered tissue culture medium 199 supplemented with 50 mg/mL Gentamycin sulfate [TCM 199B; Group II], TCM 199B supplemented with Leptin (10ng/mL) [TCM 199B+Leptin; Group III], Standard medium supplemented with Leptin (10ng/mL) [i.e., TCM 199B supplemented with T4 (1mg/mL), FSH (2.5mg/mL), IGF-I (10ng/mL), GH (1mIU/mL) is called Standard medium] [Group IV].Culture medium was pre incubated for 1hr at 39 $^{\circ}$C under humidified atmosphere of 5% $CO_2$ in the air. The selected follicles were washed thrice in the culture medium and subsequently placed individually in 20µl droplets of the culture medium in 35mm plastic culture dishes (cat. No. 153066, Nalge Nunc, Denmark). To avoid evaporation of the medium, the micro droplets were overlaid with autoclaved light weight mineral oil (Sigma M 8410) pre-equilibrated with the medium over night at 39 $^{0}$C in 5% $CO_2$ in air. These culture dishes were incubated at 39 $^{0}$C under humidified atmosphere in 5% $CO_2$ in air for up to six days. This culture procedure was found to be optimum in previous studies in the laboratory [Arunakumari *et al.,* 2010; Kona *et al.,* 2016] [3, 12]. 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.

**2.3 Immunohistochemistry assay for the detection of Leptin receptor protein (LepR)**

Leptin receptor protein (LepR) protein was detected in different stages of *in vivo* developed ovarian follicles and differently cultured preantral follicles using ultra- sensitive ABC Rabbit IgG staining kit (Catalogue No. 32054; Thermo scientifics) according to the manufacturer instructions as mentioned below.

**2.3.1 Immunohistochemistry procedure for detection of Lep R protein**

Different stages of *in vivo* grown (Group-I) and *in vitro* grown PFs' in four culture media (Groups II to IV) at different stages of development (i.e., PFs' exposed to respective culture medium for 3 min, two, four, six day cultured PFs and COCs from six day cultured PFs' after IVM) were freshly collected and subjected to IHC assay for the detection of Leptin receptor. Thus totally five *in vivo* groups (each group containing 3-8 follicles) and 15 *in vitro* groups (each group containing 3-8 follicles) were used in the present study.

Each group of follicles was taken in 35mm plastic culture dish and 50µL of PBS was added to it and incubated for 20min. Then carefully PBS was removed and 50µL of blocking buffer was added to it and incubated for 20min. Excess blocker was blotted and 50µL of primary antibody (Biorybt) was applied and incubated for 30min (For negative control, PBS was used instead of primary antibody). Follicles were then washed for 10min with PBS and 50µL of Biotinylated secondary antibody was applied and incubated for 30min. This was followed by washing of follicles for 10min with PBS before adding 50µL of ABC reagent and incubated for 30min. Follicles were washed for 10min with PBS followed by addition of 50µL of DAB as substrate and incubated for 5-7min. Then the follicles were washed for 5min with PBS and subsequently subjected to incubation in 50µL counter stain (methyl green) for 5 min. Before examining under microscope follicles were washed 8-9 times with PBS and the images were recorded by Leica DC 200 digital camera. For detection of LepR protein expression through IHC, depending on the intensity of brown colour appearance in the follicles after the final counter staining the receptor localization was divided in to three categories: 1) Mild expression (light brown) 2) Moderate expression (medium brown) and 3) Strong expression (dark brown).

## 3. Results

Immuno histochemistry analysis revealed that Leptin receptor protein (LepR) was expressed both in the oocytes and cumulus in all the development stages of follicles which were grown *in vivo* or cultured *in vitro* in three different conditions [Figure 2]. It was observed that intensity of Leptin receptor protein expressed maximum in the oocytes and mild to moderate in the surrounding cumulus cells in either *in vivo* grown (Fig.2) or *in vitro* cultured PFs' in different media. In overall the intensity of LepR protein expression was increased as the follicles develop either *in vivo* or *in vitro* towards later stages of development [Figure 2].

**3.1 Expression of Leptin receptor protein (LepR) in the cumulus cells and oocytes of *in vivo* grown ovarian follicles**

In the preantral follicles very mild expression of Leptin receptor was noted in the oocytes and surrounding cumulus cells [Figure 2: A]. Leptin receptor protein expression in the *in vivo* grown ovarian follicles in sheep was markedly observed in the oocytes of early antral, antral and large antral follicles and only to some extent in the surrounding cumulus cells in these stages of follicular development [Figure 2: B-D]. Whereas aspiration and subsequent *in vitro* maturation (IVM) of the cumulus oocyte complexes from graffian follicles resulted in maximum LepR protein expression in these oocytes than in other *in vivo* stages of follicular development [Figure 2: E]

**3.2 Expression of Leptin receptor protein (LepR) in the cumulus cells and oocytes of *in vitro* cultured preantral follicles in different culture media**

The preantral follicles were cultured separately in three conditions including TCM 199B (Group II), TCM 199B+Leptin (Group III) and Standard medium+Leptin (Group IV). Leptin receptor protein expression in the PFs' cultured in group II showed mild expression in the PFs' exposed to culture medium for 3 min [Figure 2: F] and two day [Figure 2: G] culture stages in the oocytes and surrounding cumulus cells but moderate expression of LepR protein was observed in four days [Figure 2: H] and six days [Figure 2: I] cultured PFs' in both oocytes and cumulus cells. LepR protein localization was found to be strong in the oocytes from COCs isolated from six day culture and subsequent IVM for 24hrs [Figure 2: J].

Immunolocalization of LepR in the PFs' grown in group III was mild in the PFs' exposed to culture medium for 3 min but moderate localization of LepR was observed in PFs' cultured two days [Figure 2: L], four days [Figure 2: M] and six days [Figure 2: N] culture period in both oocytes as well as the surrounding cumulus cells (Fig.). In this group also the immunopresence of LepR was strongly expressed in the oocytes from COCs isolated from six day culture and subsequent IVM for 24hrs [Figure 2: O].

Leptin receptor protein localization in the PFs' cultured in group IV was moderate in the PFs; exposed to culture medium for 3 min, two days and four days culture period [Figure 2: P-R]. But strong LepR protein expression was observed in the oocytes and cumulus cells of PFs' cultured for six days [Figure 2: S]. The immunolocalization of LepR in this group was found to be very strong in the oocytes from COCs isolated from six day culture and subsequent IVM for 24hrs [Figure 2: T].

Among the different culture conditions, exposure of PFs' to medium supplemented with Leptin (10ng/mL) i.e., groups III and IV showed better localization of LepR protein than in other groups [Figure 2: K-T]. However, supplementation of Leptin along with other growth factors to culture the PFs' resulted in strong LepR expression in both oocytes and the cumulus cells [Figure 2: P-T]. From the results it is also evident that the expression of LepR protein was found to be maximum in the oocytes from cumulus oocyte complexes collected after *in vitro* maturation of six day cultured preantral follicles in respective culture media than in other *in vitro* stages especially in the oocytes from PFs' cultured in group IV medium [Figure 2: T].

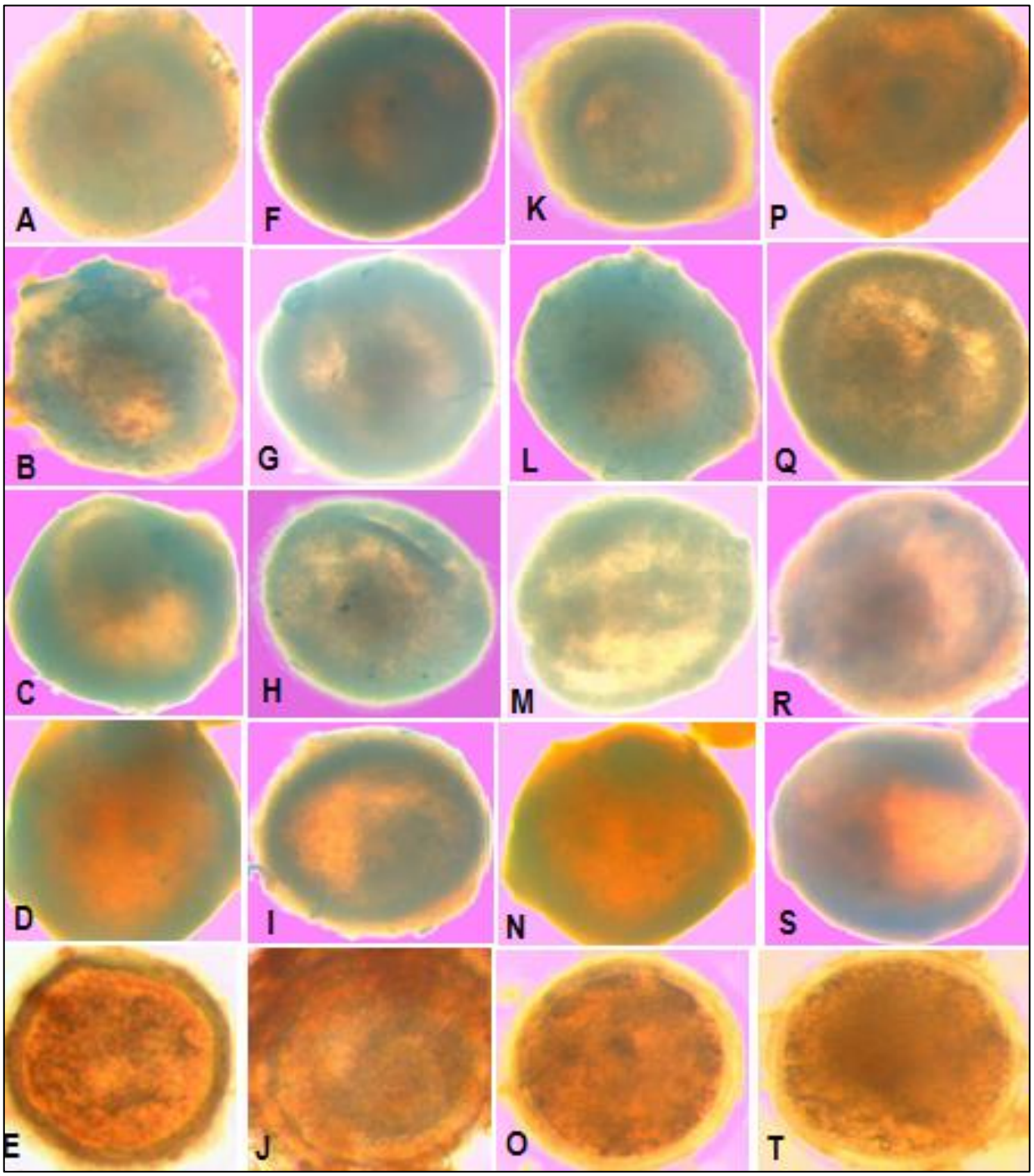


**Fig 2:** (A-Y) Different stages of *in vivo* grown ovarian follicles (PFs') and PFs' cultured in TCM199B, Standard Medium (SM), TCM199B+Kp, SM+Kp subjected to Kisspeptin receptor protein detection by Immunohistochemistry assay A: Preantral follicle, B: Early antral follicle, C: Antral follicle, D: Large antral follicle, E: Oocytes of COCs obtained from large antral follicles subjected to *in vitro* maturation for 24hrs. F, G, H, I, J: PFs' exposed to culture medium for 3 min, two day, four day, six day and Oocytes of COCs from six day cultured follicles subjected IVM for 24hrs are the different stages of PFs' cultured in TCM199B medium respectively. K, L, M, N, O: PFs' exposed to culture medium for 3 min, two day, four day, six day and Oocytes of COCs from six day cultured follicles subjected IVM for 24hrs are the different stages of PFs' cultured in TCM199B + Leptin medium respectively. P, Q, R, S, T: PFs' exposed to culture medium for 3 min, two day, four day, six day and Oocytes of COCs from six day cultured follicles subjected IVM for 24hrs are the different stages of PFs' cultured in Standard medium + Leptin respectively.

## Discussion

This is the first study demonstrating the immunohistochemical expression of Leptin receptor protein (LepR) in different stages of ovarian follicles of sheep grown *in vivo* or cultured *in vitro* in different media. The immunopresence of LepR protein was observed in the ovaries of of mouse (Ryan *et al.,* 2002) [17], rat (Ryan *et al.,* 2003) [16], rabbit (Zerani *et al.,* 2004) [19], goat (Batista *et al.,* 2013) [4], bovine (Kumar *et al.,* 2012) [13], horse (Lange-Consiglio *et al.,* 2013) [4] and humans (Abir *et al.,* 2005) [1]. However meager reports are available on the local regulatory role of Leptin in modulating the sensitivity of follicles thereby promoting better growth of follicles during culture which could be related to variations in its own receptor protein expression (Batista *et al.,* 2013) [4]. Therefore as an attempt, this study aimed at identification of LepR protein localization in

different development stages of follicles grown *in vivo* and cultured *in vitro* in various conditions. Leptin receptor (LepR) protein was expressed both in the oocytes and cumulus in all the stages of PFs' development grown *in vivo* or cultured *in vitro* in three different conditions. In our study it was observed that intensity of LepR protein expressed maximum in the oocytes and mild to moderate in the surrounding cumulus cells in either *in vivo* grown or *in vitro* cultured PFs' in all the groups under study and intensity was good in later follicular stages than in earlier stages of development. Our results coincide with that of Ryan *et al.,* (2003) [16] in rat ovaries where staining intensity was higher in the granulosa and theca cells of large follicles than those of small follicles. In contrary to our results, Ryan *et al.,* (2002) [17] in mice ovaries reported the highest intensity of staining in the thecal cells as compared to granulosa, oocytes and CL suggesting their higher receptiveness. Whereas similar to our findings Batista *et al.,* (2013) [4] observed intense staining in the cytoplasm of oocytes of both preantral and antral follicles and weak in the thecal cells. These differences in the intensity of staining might be due to species variation and method followed for protein expression. In our study we observed a progressive increase in the LepR protein expression in the follicles towards the later stages of development specifically in leptin supplemented groups. However no previous reports are available to compare the stage specific changes of Leptin receptor protein in the cumulus cells and oocytes in ovarian follicles cultured in Leptin. But overall results showed that Leptin stimulated its own receptor expression in the later stages of development in both cumulus cells and oocytes. The expression of Leptin receptor protein in the cumulus cells and oocytes was more or less similar to mRNA expression studied earlier in our laboratory (Sravani pragna *et al.,* 2020) [18]. Also significantly higher mRNA expression of Leptin receptor observed in antral and large antral stage in *in vivo* and PFs' grown in different culture media in previous studies (Dupuis *et al.,* 2014; Sravani pragna *et al.,* 2020) [18] correlate with the protein expression in present study as evidenced by IHC. Among the different groups studied the leptin receptor protein expression expressed better in the follicles cultured in group IV i.e., leptin supplemented to standard medium containing different growth factors and hormones. These results are similar to Gregoraszczuk *et al.,* (2006) [9, 10] who observed Leptin receptor expression in the granulosa cells of prepubertal pig ovaries when IGF and GH added to the culture media as compared to control media without IGF and GH. Similarly Gregoraszczuk *et al.,* (2007) reported that IGF-I had stimulatory effect on Leptin receptor expression and found 1.5 to 2 fold increase in growing follicles of pig when IGF added to the culture media. This increase might be due to additive or synergistic effects among Leptin, Growth hormone, IGF-I and Thyroxine present in standard medium on the expression of Leptin receptor. Therefore from the present results it is concluded that supplementation of Leptin @ 10ng/mL stimulated its own receptor protein expression in both cumulus cells and oocytes from early antral stage to large antral stage as well as in their corresponding *in vitro* groups and more specifically during leptin supplementation to culture media. This stimulatory effect of Leptin on its own receptor protein was further enhanced in conditioned medium containing different growth factors along with Leptin indicating the additive effects of Leptin with other growth factors and hormones.

## 5. Acknowledgment

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