RESEARCH ARTICLE Indian Journal of Animal Research, Volume 55 Issue 9: 1044-1048 (September 2021)

Leptin Supplementation Stimulates Synergism with Growth Factors and Hormones to Express Its Receptor in Cultured Preantral Follicles of Sheep

K. Sravani Pragna, V. Praveen Chakravarthi, Deepa Pathipati, B. Rambabu Naik, L.S.S. Varaprasad Reddy, B. Punya Kumari[1], A.V.N. Siva Kumar

10.18805/IJAR.B-4218

ABSTRACT

Background: Leptin receptor is a transmembrane receptor that regulates reproduction at molecular level. Since for action of any hormone on target cell and to have local action on any tissue, expression of its own receptor is necessary and also it is not known whether such improvement in ovarian follicular development by Leptin is mediated through presence of its homologous receptors in the sheep ovaries. Therefore this study aimed on expression of Leptin receptor mRNA in cultured ovarian follicles of sheep by RT PCR.

Methods: Leptin receptor mRNA expression in sheep was studied using qRT-PCR from: (i) *In vivo* grown preantral, early antral, antral, large antral follicles and cumulus oocyte complexes obtained from large antral follicles subjected to 24h of *in vitro* maturation and (ii) PFs' exposed to three different culture media for 3 min, two, four or six days and subsequently matured *in vitro* for 24h.

Result: Leptin receptor was observed at all stages ovarian follicles in both cumulus cells and oocytes. Leptin supplementation along with other growth factors and hormones stimulated the expression of its receptor mRNA which is parallel to *in vivo* stages which could suggest synergistic action of growth factors and hormones with Leptin.

Key words: Leptin, Leptin receptor, Preantral follicle, Sheep.

INTRODUCTION

Leptin, a metabolic hormone plays an important role in reproductive functions like ovarian folliculogenesis, oocyte maturation (Kamalamma *et al.,* 2016) and modulation of steroidogenesis (Anil Kumar *et al.,* 2019). Recently its effect on development of preantral follicles was studied and found that optimum dose of Leptin that supported the growth of sheep PFs' *in vitro* was 10ng/mL (Kamalamma *et al.,* 2016). It favored the expression of antiapoptotic genes and suppressed apoptotic gene during different stages of follicle development and also improved aromatase gene expression, an enzyme essential for conversion of Androgens to Estrogens (Lakshminarayana *et al.,* 2014).

Leptin receptor (LepR) is a transmembrane receptor with six isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf), of which Ob-Rb is longest form and its mRNA expression was studied in preovulatory follicles and corpus luteum (Smolinska *et al.,* 2013) indicating its ability to regulate reproduction at molecular level. Since for action of any hormone on target cell and to have local action on any tissue, expression of its own receptor is necessary and also it is not known whether such improvement in ovarian follicular development by Leptin is mediated through presence of its homologous receptors in the sheep ovaries as a local factor. Therefore present study is undertaken with an idea of understanding the evolution of Leptin receptors as the follicular development progresses from preantral follicles to ovulation stage. Moreover this will enable designing of appropriate media to culture preantral follicles by inclusion of Leptin along with different growth factors and hormones.

Department of Veterinary Physiology, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India.
[1]Department of Animal Genetics and Breeding, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India.

Corresponding Author: A.V.N. Siva Kumar, Department of Veterinary Physiology, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India. Email: priyamshiva@yahoo.com

How to cite this article: Pragna, K.S., Chakravarthi, V.P., Pathipati, D., Naik, B.R., Varaprasad Reddy, L.S.S., Kumari, B.P., Siva Kumar, A.V.N. (2021). Leptin Supplementation Stimulates Synergism with Growth Factors and Hormones to Express Its Receptor in Cultured Preantral Follicles of Sheep. Indian Journal of Animal Research. 55(9): 1044-1048. DOI: 10.18805/IJAR.B-4218.

Submitted: 15-06-2020 **Accepted:** 03-09-2020 **Online:** 28-12-2020

MATERIALS AND METHODS

All the methods employed in this study were described in detail in several earlier publications from Embryo Biotechnology Laboratory, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati. However, a brief description with respect to isolation and culture of PFs' (Arunakumari *et al.*, 2010), RNA isolation (Lakshminarayana *et al.,* 2014), reverse transcription and RT-PCR (Kona *et al.,* 2016; Srividya *et al.,* 2017; Anil kumar *et al.,* 2019) is given hereunder.

Collection of ovaries and isolation of different stages of ovarian follicles

A total of 480 ovaries collected from sheep slaughter house located in Gandhi road, Tirupati on different days during 2018 were used in this study. Ovaries recovered were transported to the laboratory within 1h after slaughter in sterile, warm (37°C) phosphate buffered saline.

In vivo grown intact preantral (PFs'), early antral, antral, large antral follicles and cumulus oocyte complexes (COCs) were mechanically isolated (Fig 1 A, C, E, G) by micro dissection method from ovarian cortex (Group I; 25 replicates) under a stereo-zoom microscope (SMZ 2T, Nikon corporation, Japan) according to the methods developed in our laboratory (Arunakumari *et al.,* 2010) and different development stages of follicles were shown in Fig 1.

Culture media

The *in vitro* groups were divided based on the type of medium used to culture the preantral follicles (PFs'). PFs' cultured in TCM 199B without any supplementation (Group II; 30 replicates), TCM 199B supplemented with 10ng/ml of ovine leptin (My Biosource Cat No. MBS142116, USA) (Group III; 30 replicates) and TCM199B supplemented with gentamycin sulphate (50µg/ml), Thyroxine (1µg/ml), Follicle stimulating hormone (2.5µg/ml), Insulin like Growth factor-1 (10ng/ml), of growth hormone (1MIU/ml) and ovine Leptin (10ng/ml) (Group IV; 30 replicates).

Selection and culture of the preantral follicles and *in vitro* maturation (IVM) of the oocytes obtained from the *in vivo* grown and cultured large antral follicles

Preantral follicles in the size range of 250-400µm with intact basement membrane were selected for the culture (Fig 1A). All the subsequent methods of culture and medium preparation were followed as in previous studies in the laboratory (Arunakumari *et al*., 2010; Kona *et al.,* 2016).

The cumulus cells and oocytes were collected from different stages of *in vivo* grown follicles (Group I; Fig 1 A, E, I, M, Q, I) and their corresponding *in vitro* stages *i.e*., PFs' cultured in Group II (Fig 1 B, F, J, N, R), Group III (Fig 1 C, G, K, O, S), Group IV (Fig 1 D, H, L, P, T) for 3min, 2, 4 or 6 day cultured follicles and COCs from 6 day cultured follicles (Fig 1 J).

Isolation and quantification of total RNA

Isolation of RNA was carried out by pooling cumulus cells and oocytes from 30-50 follicles at each development stages of follicles using patented Medox-Easy spin column Total RNA Mini prep Kits (13, 15, 16) according to the manufacturer's instructions (Medox Biotech India Pvt. Ltd., Chennai, India). Concentration and purity of RNA was determined using Nanodrop lite (Thermo scientific S.No.1354). RNA samples having purity (Absorbance at 260/280) in the range of 1.8-2.1 only were used in the expression studies. The RNA sample was stored at -70°C till analyzed.

Reverse transcription (RT) and real-time PCR

High capacity reverse transcription kit (Applied Biosystems part no: 4368814) was used for the reverse transcription. RT reaction was carried out for 10 minutes at 25°C, for 120 minutes at 37°C and for 5 minutes at 85°C in a thermocycler (Eppendorf Mastercycler Gradient) according to the manufacturer's instructions.

Quantitative expression of Leptin receptor gene was studied at different development stages of the *in vivo* grown and corresponding stages of the three groups of cultured ovarian follicles (Table 1). The geometric means of RPLPO, HPRT1 and 18SrRNA genes (17) were used as the normalizer in the analysis of Leptin receptor gene expression. The mRNA sequence for reference genes (RPLPO, HPRT1 and 18SrRNA) and target gene (Leptin receptor) were retrieved from Genbank and custom designed 20X gene expression assays mixes for these genes were obtained from Applied Biosystems (Assay on Demand lot nos: 1402507 A2, A3, A8, 1428816). The primers and probe sequence details of reference genes (RPLPO, HPRT1 and 18SrRNA) and target gene (Leptin receptor) are given in the Table 2. Real-time RT-qPCR was performed on Applied Biosystems 7500 machine. Each 25 µl reaction mix contained 12.5 µl of Taq Man Uni-versal PCR Master Mix (2x), 1.25 µl of 20X gene expression assay mixture, 10 ng of cDNA sample in nuclease free water. Thermal cycling conditions were Erase UNG (Uracil N- glycosylase) activation 2 min @ 50°C, Ampli Taq Gold DNA polymerase activation10 min @ 95°C followed by 40 cycles of 15 s @ 95°C and 1 min @ 60°C. For the calculation of the expression levels (RQ values) of different target genes, first the Ct values of target and reference genes were converted to quantity inputs by using the formula 2 minimum Ct-sample Ct.

Statistical analysis

The entire experiment of Leptin receptor gene expression was repeated twice and the data were pooled after Barlett's test confirmed the homogeneity of variances. Log RQ values were analysed by two-way ANOVA with unequal number of observations followed by Tukey multiple comparisons test (SPSS version 20). P values $\leq$ 0.05 were considered significant.

RESULTS AND DISCUSSION

This is the first report on the Leptin receptor gene expression during different development stages of sheep ovarian follicles grown *in vivo* or preantral follicles (PFs') cultured *in vitro* in three different conditions. A total 2460 follicles were isolated to conduct this study. Leptin receptor gene was expressed in both cumulus cells and oocytes collected from all the groups (Table 1). In group I, cumulus cells from large antral follicles showed highest expression with an increased trend from early antral to large antral follicles. These results are similar to findings of Gregoraszczuk *et al.* (2006) in porcine ovary who reported 6 folds increase in the Leptin receptor expression in antral follicles than early antral follicles, indicating a possible involvement of Leptin in

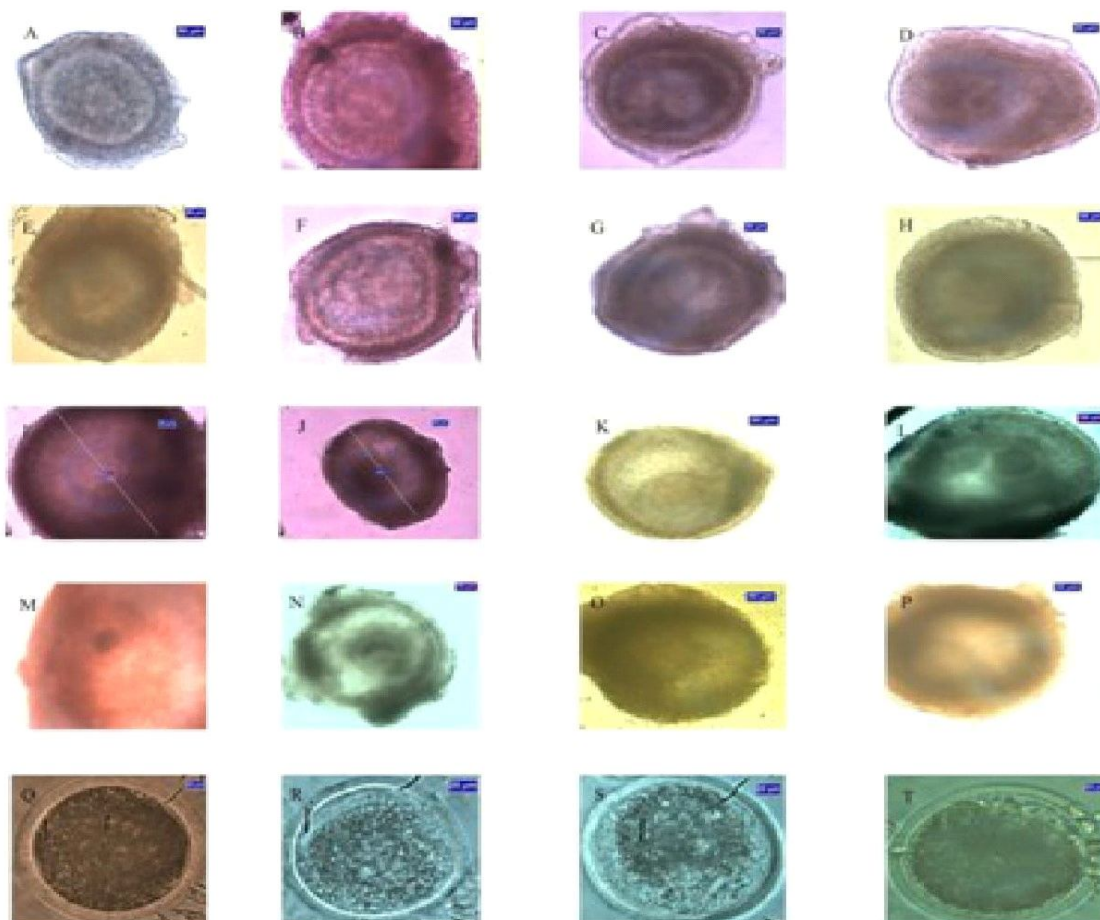

Fig 1: Development of stages of the *in vivo* grown and corresponding stages of cultured ovarian follicles in different media.
Group I: A: Preantral follicle, E: Early antral follicle, I: Antral follicle, M: Large antral follicle, Q: Oocytes of COCs obtained from large antral follicles subjected to *in vitro* maturation for 24h.
B, F, J, N, R: 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 24h are the different stages of PFs' cultured in group II respectively.
D, H, L, P, 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 24h are the different stages of PFs' cultured in group III respectively.
C, G, K, O, S: 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 24h are the different stages of PFs' cultured in group IV respectively.

follicular development. Batista *et al.* (2013) also found that Leptin receptor mRNA expression levels were higher in the granulosa cells of large antral follicles than corresponding cells from small antral follicles. In our laboratory recent study indicated that estradiol concentration followed an amplified fashion from PFs' to large antral follicles in *in vivo* grown sheep ovarian follicles (Anil kumar *et al.,* 2019). In this study also the pattern of expression of Leptin receptor gene in the cumulus cells and oocytes from the PFs' cultured in group II appeared to be similar to gene expression of aromatase, an enzyme essential for synthesis of estrogen hormone (Lakshminarayana *et al.,* 2014). Therefore increase in the expression of Leptin receptor in later stages of follicular development than early stages might be an influence of estrogen hormone on Leptin receptor expression (Meli *et al.,* 2004; Chen *et al.,* 2006; Garofalo *et al.,* 2006).

Leptin receptor gene was expressed in all the stages both in the cumulus cells and oocytes when Leptin was added to culture medium (Table 1). In group III Leptin receptor gene expression was down regulated in PFs' exposed to 3 min and 2 day cultured follicles and expression was similar in four day and six day cultured PFs' in cumulus cells as compared to group-II. While in oocytes of PFs' cultured in group III the expression was upregulated as compared to the group II grown follicles except oocytes in PFs' exposed to medium for 3 min. However, decreased expression in early stages and upregulated expression of Leptin receptor at later stages of both cumulus cells and oocytes in PFs' grown in group III could be due to differential action of Leptin on its own receptor expression as reported by cordova *et al.* (2011).

The expression levels in PFs' grown in group III and group IV were almost similar but higher when compared to

Table 1: Expression of Leptin receptor gene in cumulus cells and oocytes in *in vivo* and *in vitro* grown follicles.

Source	Leptin receptor gene expression (log_{10} Relative quantification)				
In vivo			*In vitro*		
	(Group I)		Group II	Group III	Group IV
Cumulus cells from					
Preantral follicles	0.59 ± 0.12^{1a}	PFs' exposed to medium for 3 minutes	1.33 ± 0.03^{1b}	0.47 ± 0.46^{1a}	1.19 ± 0.05^{1a}
Early antral follicles	0.01 ± 0.008^{2a}	PFs' cultured *in vitro* for two days	1.00 ± 0.22^{1b}	0.20 ± 0.12^{1a}	0.57 ± 0.03^{2a}
Antral follicles	0.68 ± 0.09^{1a}	PFs' cultured *in vitro* for four days	0.58 ± 0.15^{2b}	0.49 ± 0.07^{1b}	0.79 ± 0.01^{3a}
Large antral follicles	1.45 ± 0.15^{3a}	PFs' cultured *in vitro* for six days	1.29 ± 0.48^{1b}	1.30 ± 0.32^{2b}	1.46 ± 0.02^{4a}
Cumulus cells from COCs after IVM	0.81 ± 0.0008^{4a}	COCs of 6 day cultured PFs' matured *in vitro* for 24 h	0.43 ± 0.03^{2c}	0.07 ± 0.29^{1c}	2.06 ± 0.04^{5b}
Oocytes from					
Preantral follicles	1.66 ± 0.07^{1a}	PFs' exposed to medium for 3 minutes	1.74 ± 0.05^{1a}	1.01 ± 0.11^{1b}	0.88 ± 0.02^{1b}
Early antral follicles	0.31 ± 0.01^{2a}	PFs' cultured *in vitro* for two days	0.38 ± 0.30^{2a}	1.35 ± 0.23^{1b}	0.74 ± 0.15^{1a}
Antral follicles	0.42 ± 0.01^{2a}	PFs' cultured *in vitro* for four days	0.31 ± 0.13^{2a}	1.52 ± 0.24^{1b}	1.95 ± 0.02^{2b}
Large antral follicles	1.58 ± 0.02^{1a}	PFs' cultured *in vitro* for six days	0.74 ± 0.03^{3b}	1.58 ± 0.13^{1a}	1.73 ± 0.14^{2a}
Oocyte from COCs after IVM	0.0 ± 0.02^{3a}	COCs of 6 day cultured PFs' matured *in vitro* for 24 h	0.17 ± 0.07^{2a}	1.75 ± 0.52^{1b}	1.22 ± 0.10^{1b}

Values with different numeric superscripts in a column within *in vivo* or *in vitro* stages and different alphabetic superscripts between corresponding *in vivo* and *in vitro* stages are significantly different ($P\leq0.05$).

group I or group II (Table 1). We observed that LepR expression was highest in the cumulus cells from COCs after IVM and oocytes from preantral follicles cultured for four days in group IV as compared to all other groups (Table 1). The expression levels of Leptin receptor of preantral follicles (PFs') grown in group I and IV was similar in all stages of development except COCs after *in vitro* maturation which was higher in group IV (Table 1). Such significantly higher expression of Leptin receptor in antral and large antral follicular stages of PFs' grown in group IV could be due to synergistic effects among Leptin, IGF, GH, FSH and Thyroxine in the culture medium (Gregoraszczuk *et al.,* 2006). In both cumulus cells and oocytes the Leptin receptor gene was down regulated from preantral stage to early antral stage in all the groups, which might be due to less requirement of Leptin signalling mechanism at this particular stage of transition. Thus supplementation of Leptin along with other hormones or growth factors stimulated its receptor gene expression better than *in vivo* or other media used to culture the preantral follicles.

CONCLUSION

It is concluded that Leptin stimulated its own receptor expression in both cumulus cells and oocytes from early antral stage to large antral stage. Further this stimulatory effect of Leptin on its own receptor showed synergism in the preantral follicles cultured in medium containing leptin along with other growth factors and hormones.

ACKNOWLEDGEMENT

This work was supported by a research grant from the council of Scientific and Industrial Research (grant 37 (1612)/13/EMR-II) to A.V.N. Siva Kumar.

REFERENCES

Anil Kumar, P., Siva Kumar, A.V.N., Deepa, P., Praveen Chakravarthi, V., Brahmaiah, K.V. and Rao, V.H. (2019). Leptin induced *in vitro* development of ovarian follicles in sheep is related to the expression of P450 aromatase and steroidiogenesis. Theriogenology. 136: 1-6.

Arunakumari, G., Shanmugasundaram, N. and Rao, V.H. (2010). Development of morulae from the oocytes of cultured sheep preantral follicles. Theriogenology. 74: 884-894.

Batista, A.M., Silva, D.M., Rêgo, M.J, Silva, F.L, Silva, E.C, Beltrao, E.I, Gomes Filho, M.A, Wischral, A. and Guerra, M.M. (2013). The expression and localization of leptin and its receptor in goat ovarian follicles. Animal Reproduction Science. 141: 142-147.

Chen, H.P., Fan, J. and Cui, S. (2006). Detection and estrogen regulation of leptin receptor expression in rat dorsal root ganglion. Histochemistry and Cell Biology. 126: 363-369.

Córdova, B., Morató, R., de Frutos, C., Bermejo-Álvarez, P., Paramio, T., Gutiérrez-Adán, A. and Mogas, T. (2011). Effect of leptin during *in vitro* maturation of prepubertal calf oocytes: Embryonic development and relative mRNA abundances of genes involved in apoptosis and oocyte competence. Theriogenology. 76: 1706-1715.

Garofalo, C., Koda, M., Cascio, S., Sulkowska, M., Kanczuga-Koda, L., Golaszewska, J., Russo, A., Sulkowski, S. and Surmacz, E. (2006). Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: Possible role of obesity-related stimuli. Clinical Cancer Research. 12: 1447-1453.

Gregoraszczuk, E.L., Ptak, A., Wojciechowicz, T. and Nowak, K. (2006). Action of IGF-I on expression of the long form of the leptin receptor (ObRb) in the prepubertal period and throughout the estrous cycle in the mature pig ovary. Journal of Reproduction and Development. 53: 289-295.

Kamalamma, P., Kona, S.S.R., Praveen Chakravarthi, V.P, Siva Kumar, A.V.N., Punyakumari, B. and Rao, V.H. (2016). Effect of Leptin on *in vitro* development of ovine preantral ovarian follicles. Theriogenology. 85: 224-229.

Kona, S.S.R., Chakravarthi, V.P., Siva Kumar, A.V.N., Srividya, D., Padmaja, K. and Rao, V.H. (2016). Quantitative expression patterns of GDF9 and BMP15 genes in sheep ovarian follicles grown *in vivo* or cultured *in vitro*. Theriogenology. 85: 315-322.

Lakshminarayana, B.N.V., Chakravarthi, V.P., Brahmaiah, K.V. and Rao, V.H. (2014). Quantification of P450 aromatase gene expression in cultured and *in vivo* grown ovarian follicles in sheep. Small Ruminant Research. 117: 66-72.

Meli, R., Pacilio, M., Raso, G.M., Esposito, E., Coppola, A., Nasti, A., Di Carlo, C., Nappi, C. and Di Carlo, R. (2004). Estrogen and raloxifene modulate leptin and its receptor in hypothalamus and adipose tissue from ovariectomized rats. Endocrinology. 145: 3115-3121.

Smolinska, N., Kaminski, T., Siawrys, G. and Przala, J. (2013). Expression of leptin and its receptor genes in the ovarian follicles of cycling and early pregnant pigs. Animal. 7: 109-117.

Srividya, D., Praveen Chakravarthi, V., Kona, S.S.R., Siva Kumar, A.V.N., Brahmaiah, K.V. and Rao, V.H. (2017). Expression of kit ligand and insulin like growth factor binding protein 3 during *in vivo* or *in vitro* development of ovarian follicles in sheep. Reproduction in Domestic Animals. 52: 661-671.