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Research Article
Supplementation of Leptin on *in vitro* Maturation of Sheep Oocytes

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Abstract

Background and Objective: Leptin has beneficial effects on mammalian oocyte maturation, development and enhances the transcription levels of developmentally important genes. The aim of present study was to determine the factors affecting *in vitro* maturation of sheep oocytes. **Materials and Methods:** Ovaries from slaughtered sheep were collected from local Abattoir of Hyderabad (India). Four experiments were conducted. Experiment I: Efficacy of oocyte retrieval methods in relation to oocyte quality and quantity were studied. Experiment II: Effect of season on sheep oocytes quality and numbers were recorded. Experiment III: Determine the effect of presence of Corpus Luteum (CL) on oocyte yield. In experiment IV: Studied effect of leptin addition on maturation media to compare the IVM (*In vitro* maturation) status like cumulus-oocyte complexes expansion and M-II stage of sheep oocytes in combination with traditional *in vitro* maturation medium. Oocytes were cultured with different concentration of leptin. T_1: 10, T2: 20, T_3: 50, T_4: 100 ng mL^{-1} and T5: Control incubated at 39°C in 5% CO_2 under humidified atmosphere for 24 h. **Results:** Total 3241 oocytes were retrieved from 1520 sheep ovaries. The percentage of good quality oocytes was higher in the aspiration method (65.8%) as compared to the slicing (54.2%) and post aspiration slicing method (36.1%). The oocytes recovery recorded during spring (March-April) was significantly higher as compared to winter and summer. On an average 1.88±0.14 oocytes per ovary were recovered from ovaries without CL as compared to ovaries with CL 1.37±0.1. The leptin 20 ng mL^{-1} shows Cumulus-Oocyte Complexes (COC) expansion (76.80±1.59) and polar body extrusion (50.63±3.94) whereas, leptin-20 ng mL^{-1} with IVM medium also exhibited Cumulus-Oocyte Complexes (COC) expansion (81.90±1.87) and polar body extrusion (56.87±2.92). Leptin 20 ng mL^{-1} with IVM medium shows best result on *in vitro* maturation of sheep oocytes. **Conclusion:** This study findings suggested that aspiration method was best for oocyte retrieval. Quality and numbers was better on spring (March-April) season as compare to winter and summer. The oocyte yield was significantly lower on ovary with CL. Supplementation of leptin 20 ng mL^{-1} with IVM shows best result on *in vitro* maturation of sheep oocytes as compare leptin alone and control.

Key words: Leptin, oocytes, IVM, aspiration, post aspiration slicing

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In vitro embryo production (IVEP) is a multistep process involving oocyte maturation, fertilization and embryo culture *in vitro*. *In vitro* maturation (IVM) of oocytes provides an excellent opportunity for getting cheap and abundant embryos which will be used for carrying out basic study. Leptin is a peptide hormone that is secreted mainly by white adipocyte and plays a crucial role in regulating energy expenditure[1]. It has been implicated in the regulation of ovarian function, oocyte maturation and pre-implantation embryo development in a variety of animal species[2,3]. Many researchers showed the beneficial effect of leptin supplementation on oocyte maturation *in vitro* which enhanced meiotic progression of oocytes in bovine[4-6], pig[7,8] rabbit[9] and horse[10].

Gabr *et al.*[11] studied that 20 ng mL^{-1} of leptin increased *in vitro* nuclear maturation rate of immature oocytes their fertilization rate and blastocyst production in camel oocytes. On the other hand, the success of *in vitro* maturation of livestock species depends on many factors including oocyte retrieval methods[12] , presence or absence of Corpus Luteum (CL)[13] and season influences the quantity and quality of recovered oocytes per ovary[14].

As per the literature, there is no study about leptin addition on *in vitro* maturation of sheep oocytes in India, so with this background, the present study was conducted to explore the effect of oocyte retrieval methods, seasons, presence of corpus luteum on oocyte yield and effect of leptin addition (10, 20, 50 and 100 ng mL^{-1}) on maturation media to compare the *in vitro* maturation (IVM) status like Cumulus-Oocyte Complexes (COC) expansion and M-II stage of sheep oocytes in combination with traditional *in vitro* maturation (IVM) medium.

MATERIALS AND METHODS

Sheep ovaries were collected from government approve slaughter house at Ziaguda, Hyderabad immediately after slaughter. The ovaries were washed with PBS and transported to the laboratory within 5-6 h after collection in a thermos flask containing warm water (37°C). On reaching the laboratory, working area was cleaned with 70% alcohol and the ovaries were handled aseptically. The oocytes were collected aseptically from the ovaries by slicing, aspiration and post aspiration slicing method. On aspiration the visible follicles present on the surface of the ovary were aspirated with 20 G needle fixed to 5 mL disposable syringe containing 2 mL of handling media. By slicing, the ovaries were held firmly with the help of forceps and were sliced into possible thin sections with a BP blade and washed down with a fine jet of handling media after slicing. The petriplate containing oocytes were placed for 5 min. For post aspiration slicing method the visible follicles present on the surface of the ovary were aspirated with 20 G needles fixed to 5 mL disposable syringe containing 2 mL of handling media. The aspirated ovaries were further subjected to slicing method in order to obtain the residual oocytes present in ovary. The oocytes were graded under a stereo zoom microscope and classified according to Rahman *et al.*[15]:

Grade A: Cumulus-Oocyte Complex (COC) with four or more layers of compact cumulus cells surrounding the zona pellucida with evenly granulated cytoplasm (Fig. 1)

Grade B: The COC with 1-3 layers of compact cumulus cells surrounding the zona pellucida with evenly granulated cytoplasm (Fig. 2)

Grade C: Oocyte with fibrous cumulus layers surrounding the zona pellucida (Fig. 3)

Grade D: Oocyte without cumulus cells and an irregular ooplasm (Fig. 3)

Only grades A and B oocytes were selected and subjected to *in vitro* maturation (IVM).

Experimental design: A series of experiments were designed and carried out to standardize the oocyte retrieval method, effect of season on sheep oocytes quality and quantity, effect of presence of corpus luteum on oocytes yield and optimum concentrations of leptin in culture medium to support growth and *in vitro* maturation of sheep oocytes:

Experiment I: Oocytes were retrieved by three methods slicing, aspiration and post aspiration slicing method. Studied the efficacy of oocyte retrieval methods in relation to oocyte quality and quantity

Experiment II: The sheep ovaries were collected from slaughter house in different seasons and studied oocytes yield as well as oocyte retrieved per ovary

Experiment III: The oocytes were classified on the basis of presence or absence of Corpus Luteum (CL) on ovary. Studied the effect of CL on oocyte quality and quantity

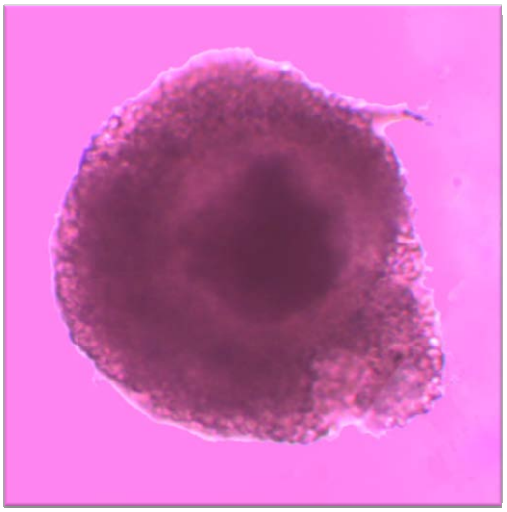

Fig. 1: Immature grade A oocyte

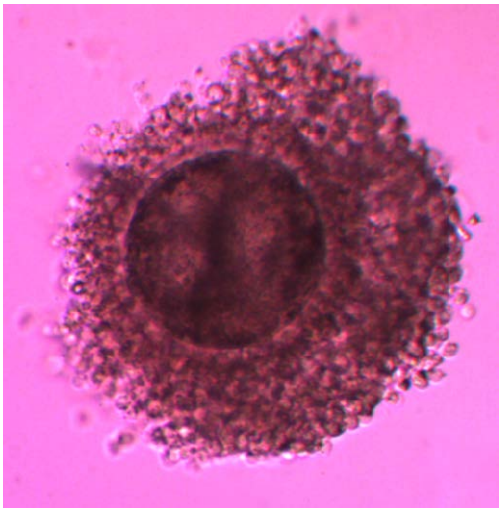

Fig. 2: Immature grade B oocyte

Experiment IV: The effect of leptin (10, 20, 50 and 100 ng mL^{-1}) on maturation media to compare the *in vitro* maturation (IVM) status like Cumulus-Oocyte Complexes (COC) expansion and M-II stage of sheep oocytes in combination with traditional *in vitro* maturation (IVM) medium

The oocytes were washed 2 times with control medium separately. Only grade A and B oocytes were allowed to mature in 50 µL droplets of maturation medium in 35 mm petridishes. The droplets were overlaid with autoclaved and pre-equilibrated light weight mineral oil and kept for 24 h in

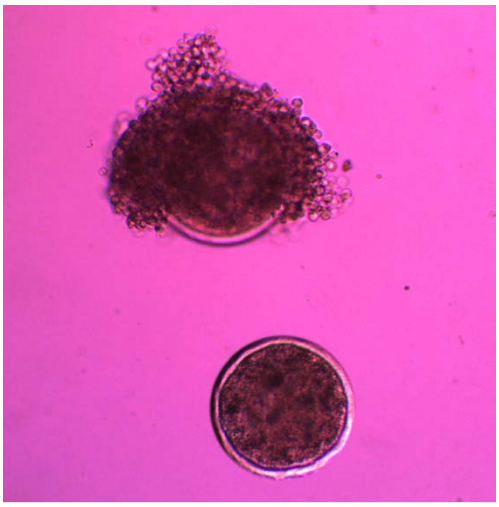

Fig. 3: Immature grade C and D oocytes

a CO_2 incubator maintained at 39°C, 5% CO_2 and high humidity. A total 110 oocytes (Batches of 10 oocytes) were used, respectively in each group. In experiment I, the oocytes were subjected to *in vitro* maturation for 24 h in different concentration of leptin (0, 10, 20, 50 and 100 ng mL^{-1}) with TCM-199 where as for experiment II, TCM-199 supplemented with 10% FBS, 10 µg mL^{-1} FSH, 10 IU mL^{-1} LH, 1 µg mL^{-1} and estradiol-17 β, 50 µg mL^{-1}. Gentamicin sulphate in different groups i.e., group I (Without leptin), group II (Leptin 10 ng mL^{-1}), group III (Leptin 20 ng mL^{-1}), group IV (Leptin 50 ng mL^{-1}) and group V (Leptin 100 ng mL^{-1}), respectably. The assessment of maturation was done by the degree of expansion of Cumulus-Oocyte Complexes (COC) and extrusion of first polar body (PB1) into perivitelline space (Fig. 4, 5).

Statistical analysis: Data on Cumulus-Oocyte Complexes (COC) expansion and extrusion of polar body were analyzed using software package used for statistical analysis (SPSS) (Version 17). One-way ANOVA between treatment groups. Differences among treatment groups were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

A total 1520 sheep ovaries were procured from slaughter house. From those ovaries 3241 oocytes were retrieved by three methods like slicing, aspiration and post aspiration slicing method. A series of experiments were conducted to evaluate the efficacy of oocyte retrieval methods in relation to oocyte quality, effect of season on sheep oocytes quality and

quantity, presence of corpus luteum on oocytes yield and compare the *in vitro* maturation (IVM) status of oocytes in different concentration of leptin 10, 20, 50 and 100 ng mL^{-1} alone and in combination with traditional IVM medium.

Efficacy of oocyte retrieval methods in relation to oocyte quality: A total 3241 oocytes were retrieved by three methods like slicing, aspiration and post aspiration slicing method. The percentage of good quality oocytes was higher in the aspiration method (65.8%) as compared to the slicing (54.2%) and post aspiration slicing method (36.1%) (Table 1).

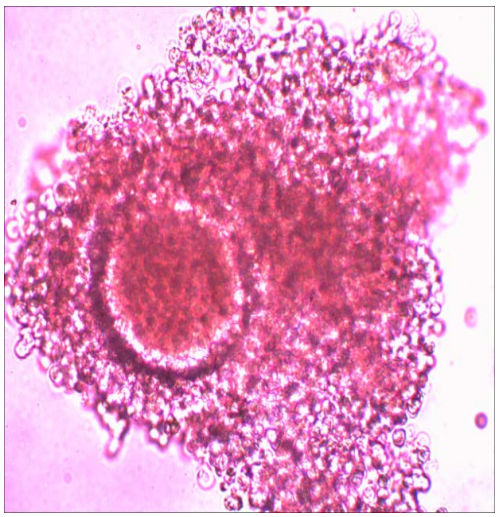

Fig. 4: Cumulus-Oocyte Complexes (COC) expansion in matured oocyte quantity, presence of corpus luteum on oocytes yield and compare the *in vitro* maturation (IVM) status of oocytes in different concentration of leptin 10, 20, 50 and 100 ng mL^{-1} alone and in combination with traditional IVM medium

Evaluation of seasonal effect on oocytes recovery: The highest number of oocytes was recovered in the spring (March-April) season 1980 followed by winter (January-February) season 766 and in summer (May-June) season 495. Oocytes retrieved per ovary was highest in spring season 4.2±0.12 followed by winter season 3.35±0.14 and lower in summer season 3.1±0.10 (Table 2).

Effect of presence of Corpus Luteum (CL)on oocyte yield: The effect of presence of corpus luteum on oocyte yield was studied. Total 311 ovaries without CL and 104 ovaries with CL yields 586 oocytes and 143 oocytes, respectively. On an average 1.88±0.14 oocytes per ovary were recovered from ovaries without corpus luteum as compare to ovaries with corpus luteum 1.37±0.11. The presence of corpus luteum on the ovary at the time of oocyte recovery significantly affected the quantity of oocytes (Table 3).

Effect of different concentration of leptin on *in vitro* maturation of sheep oocytes: The Cumulus-Oocyte

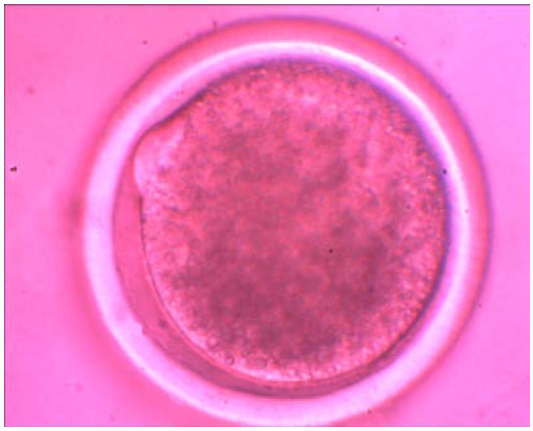

Fig. 5: First polar body extrusion into perivitelline space

Table 1: Efficacy of oocyte retrieval methods in relation to oocyte quality

Methods	No. of ovaries	No. of oocytes retrieved	Oocytes retrieved per ovary	No. of grade A and B oocytes	Grade A and B oocytes recovery (%)
Slicing	456	1083	2.40±1.24^{b}	588.0±39.04^{b}	2.18±0.17^{b} (54.2)
Aspiration	987	2053	2.08±1.23^{a}	1351.0±105.43^{a}	5.40±0.29^{a} (65.8)
Both (Post aspiration and slicing)	77	105	1.36±0.27^{c}	39.0±2.95^{c}	1.94±0.11^{c} (36.1)

Different superscripts within a column for an attribute differ significantly by Duncan's multiple range test ($p<0.05$)

Table 2: Seasonal effect on oocytes recovery

Seasons	No. of ovaries	No. of oocytes	Oocytes retrieved per ovary (Mean±SE)	Oocyte quality (Mean±SE)		
				Grade A	Grade B	Grade C
Winter	456	766	3.35±0.14^{b}	335.3±5.7^{b}	245.48±2.1^{b}	86.0±5.2^{b}
Spring	750	1980	4.20±0.12^{a}	506.0±4.4^{a}	353.30±2.3^{a}	145.0±2.7^{a}
Summer	314	495	3.10±0.10^{c}	180.0±5.5^{c}	143.28±1.7^{c}	53.1±2.6^{c}

Different superscripts within a column for an attribute differ significantly by Duncan's multiple range test ($p<0.05$)

Table 3: Effect of presence of corpus luteum on oocyte yield

Type of ovaries	No. of ovaries	No. of oocytes retrieved	Average No. of oocytes retrieved per ovary
Without corpusluteum	311	586	1.88±0.14[a]
With corpusluteum	104	143	1.37±0.11[b]

Different superscripts within a column for an attribute differ significantly by Duncan's multiple range test (p<0.05)

Table 4: Effect of different concentration of leptin on *in vitro* maturation of sheep oocytes

Concentration of leptin (ng mL^{-1})	Replicates/ No. of oocytes	Cumulus-Oocyte Complexes (COC) (Mean±SE)	M-II (Mean±SE)
10	10/110	67.74±1.94[b]	42.58±3.38[b]
20	10/110	73.80±1.59[a]	50.63±3.94[a]
50	10/110	44.47±2.59[c]	30.10±2.3[c]
100	10/110	46.28±2.89[c]	32.86±2.11[c]
Control	10/110	40.20±2.08[c]	26.71±2.38[c]

Different superscript within the column are significantly different one-way ANOVA followed by Duncan's multiple range test (p<0.05)

Table 5: Effect of different concentration of leptin combination with IVM medium on *in vitro* maturation of sheep oocytes

Concentration of leptin (ng mL^{-1})	Replicates/ No. of oocytes	Cumulus-Oocyte Complexes (COC) (Mean±SE)	M-II (Mean±SE)
10	10/110	76.59±2.30[b]	40.99±3.11[b]
20	10/110	81.90±1.87[a]	56.87±2.92[a]
50	10/110	61.24±1.77[c]	33.97±2.19[c]
100	10/110	58.57±2.86[c]	29.24±2.18[c]
Control (only IVM)	10/110	51.20±1.77[c]	28.99±2.39[c]

Different superscript within the column are significantly different one way ANOVA followed by Duncan's multiple range test (p<0.05)

Complexes (COC) expansion (73.80±1.59) was significantly higher in leptin 20 ng mL^{-1} as compared with other concentrations 10 ng mL^{-1} (67.74±1.94), 50 ng mL^{-1} (44.47±2.59) and 100 ng mL^{-1} (46.28±2.88) of leptin and polar body extrusion was significantly higher in leptin 20 ng mL^{-1} (50.63±3.94) as compared with other concentrations leptin 10 ng mL^{-1} (42.58±3.38), 50 ng mL^{-1} (30.10±2.31) and leptin 100 ng mL^{-1} (32.86±2.11) (Table 4).

Effect of different concentration of leptin in combination with IVM medium on *in vitro* maturation of sheep oocytes: The Cumulus-Oocyte Complexes (COC) expansion was highest in leptin 20 ng mL^{-1} with IVM medium (81.90±1.87) when compared with other concentrations 10 ng mL^{-1} (76.59±2.30), 50 ng mL^{-1} (61.24±1.77) and 100 ng mL^{-1} (58.58±2.86) of leptin with IVM medium. The oocytes cultured in leptin 20 ng mL^{-1} (56.87±2.92) exhibited significantly higher percent of polar body extrusion, when compared with other concentrations 10 ng mL^{-1} (40.99±3.11), 50 ng mL^{-1} (33.97±2.19) and 100 ng mL^{-1} (29.24 ±2.18) of leptin with IVM medium (Table 5).

DISCUSSION

Oocyte maturation is an essential physiological event for fertilization of oocytes in the present study, grade A and B quality oocytes recovery rate was higher in aspiration method (65.8%) followed by slicing method (54.25%) and post aspiration slicing (36.1%), the present study corroboration with the Majeed *et al.*[12]. In present study, aspiration method was best it might be due to matured follicles were used for oocytes retrieval and less number of grade A and B quality oocytes recovered in slicing method may be due to isolation of oocytes from immature follicles. These reports also confirm the report of Pawshe *et al.*[16]. The grade A and B oocytes were retrieved greatly from aspiration technique and dissection technique when compared to grade C and D oocytes. However, the present results were not in accordance with Petrean *et al.*[17] they observed that recovery of oocytes using the slicing technique in both ewes and lambs yielded more good quality oocytes per ovary than aspiration.

The present study advocated that there are significant differences between the three seasons that consequently affected the quality and the yield of oocytes that were comparable with the findings observed by Rust *et al.*[14] with regard to number of oocytes recovered , oocyte recovery rate and oocyte quality. There was a tendency for the number of grade A oocytes to be higher during the cooler months and lower during the hotter months. The findings were in accordance with Zoheir *et al.*[18] with regard to oocyte recovery and quality. In present study, quality and the yield of oocytes was low in summer season it may be due to the general tendency for a lower ovarian follicle density (Growth) during the warmer (Summer) months and after that a higher follicular population during the colder (Winter) months. Low follicular population during summer months might be due to day-light length is increased and poor nutrition.

The effect of presence of corpus luteum on oocyte yield was studied. In this study, luteal phase ovaries (With CL) yielded significantly lower numbers of oocytes 1.37±0.11 compared to non-luteal phase (Without CL) 1.88±0.14. The cause of a low number of visible follicles per ovary which were subjected to oocyte recovery by means of aspiration technique may be attributed to the fact that a large body of CL may inhibit the growth of follicles on the ovary and increase their atresia[19]. The present findings were in accordance with Davachi *et al.*[20] also observed that the presence of CL on ovaries lead to decrease in the quality and the quantity of oocytes.

However, the present results were not in accordance with Sahoo *et al.*[21] they observed that the number of buffalo

oocytes per ovary were 1.7±0.1 and 1.5±0.1 obtained from ovaries without CL and with CL, respectively.

In the present study Cumulus-Oocyte Complexes (COC) expansion (73.80±1.59) and meiotic resumption and development to M-II stage (50.63±3.94[a]) exhibited significantly higher in leptin 20 ng mL^{-1} as compared to 10, 50 and 100 ng mL^{-1} of leptin. It may be due to leptin at this concentration was found to significantly increase stimulated progesterone, estradiol, production/secretion by cultured oocyte in a dose-dependent manner with higher concentrations of leptin inhibiting growth[22]. The present results were in accordance with Gabr *et al.*[11]. They reported supplementation of leptin to maturation medium (TCM-199) at a level 20 ng mL^{-1} increased *in vitro* nuclear maturation rate of oocytes, their fertilization rate and blastocyst production in camel oocytes. Similarly, Duggal *et al.*[23] reported with regard to high doses of leptin may have inhibitory effect on ovulations but did not influence steroid levels. However, present results were not in accordance with Craig *et al.*[7] in pig, Boelhauve *et al.*[24], Paula-Lopes *et al.*[25] and Jia *et al.*[26] in bovine, Arias-Alvarez *et al.*[9] in rabbit, Khaki *et al.*[27] in buffalo, when the antral oocytes were matured in the medium supplemented with leptin at concentration 10 ng mL^{-1}. This may be due to species variation, seasonal variation, concentration of leptin supplemented and stage of follicular development at the time of oocyte collection.

Effect of different concentration of leptin in combination with IVM medium on *in vitro* maturation of sheep oocytes. The Cumulus-Oocyte Complexes (COC) expansion was highest in leptin 20 ng mL^{-1} (81.90±1.87) when compared with other concentrations 10 ng mL^{-1} (76.59±2.30), 50 ng mL^{-1} (61.24±1.77) and 100 ng mL^{-1} (58.57±2.86) of leptin with IVM medium. This may be due to up-regulation of leptin receptors at 20 ng mL^{-1}. The oocytes cultured in leptin 20 ng mL^{-1} (56.87±2.92) exhibited significantly higher percent of polar body extrusion when compared with other concentrations 10 ng mL^{-1} (40.99±3.11), 50 ng mL^{-1} (33.97±2.19) and 100 ng mL^{-1} (29.24±2.18) of leptin with IVM medium. These observations were in accordance with Singh *et al.*[28] leptin at the concentration of 20 ng mL^{-1} in maturation medium significantly ($p<0.05$) improved maturation rate, cleavage, morula and blastocyst development in buffaloes oocytes. In present study, leptin in combination with IVM medium showed best result, it may be due to the *in vitro* maturation (IVM) medium supplemented with 10% bovine serum albumin to prevent the zona pellucida hardening provides a source of albumin that balances the osmolarity and acts as a free radical scavenger. Whereas, gonadotropins and other growth factors helpful in achieving nuclear and cytoplasmic maturation it induce major alteration in the protein profile of oocytes[29].

However, present results were not in accordance with Cordova *et al.*[30] observed that oocytes matured in the presence of 1000 ng mL^{-1} of leptin showed significantly higher percentage of tunel positive cells (6.9%) than matured in Fetal Calf Serum (FCS) (5.0%) and leptin receptor (LEPR) mRNA levels were significantly higher in the group matured with FCS. This may be due to variation in concentration of leptin supplemented and species.

CONCLUSION

It was concluded that on oocyte retrieval method percentage of grade A and B quality oocytes was higher in the aspiration method. On spring (March-April) season sheep oocytes quality and numbers was better as compare to winter and summer. The oocyte yield was significantly lower on ovary with corpus luteum. On *in vitro* development of sheep oocytes could be significantly improved through addition of leptin in culture media. Supplementation of leptin 20 ng mL^{-1} with IVM shows best result as compare to leptin alone and control.

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