TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 273-283
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doi:10.3906/vet-1907-72

Effect of supplementing additives in leptin-enriched maturation medium during in vitro maturation and vitrification of goat oocytes

Reema TALUKDAR[1,*], Ranjan Kumar BISWAS[1], Govindasamy KADIRVEL[2], Bharat Chandra DEKA[1], Sudip SINHA[1], Lukumoni BURAGOHAIN[3], Birina BORA[4]

[1]Department of Animal Reproduction, Gynecology, & Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, India

[2]Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Umiam, Meghalaya, India

[3]Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, India

[4]Department of Mathematical Science, University of Delhi, Delhi, India

Received: 18.07.2019 • **Accepted/Published Online:** 26.01.2020 • **Final Version:** 06.04.2020

Abstract: The present study aimed to evaluate the effect of supplementing epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), fetal bovine serum (FBS), and bovine serum albumin (BSA) in different combinations in a maturation medium (MM) comprising TCM-199 + 5 µg/mL 17-β estradiol + 5 µg/mL ovine-luteinizing hormone (oLH) + 5 µg/mL pure follicle-stimulating hormone (pFSH) + 100 µM/mL cysteamine containing the best concentration of leptin among 10/20/25/30 ng/mL on in vitro maturation (IVM) and subsequent vitrification of matured goat oocytes. The IVM of oocytes was carried out in a CO_2 incubator for 27 h at 38.5 °C. The percentage of oocytes with cumulus expansion and polar body extrusion was found to be highest in 25 ng/mL leptin at 47.37% and 15.63%; rates of 66.67% and 28.57% were obtained when supplemented with 50 ng/mL IGF-I + 10 ng/mL EGF in medium containing 25 ng/mL leptin as compared to the other media. The in vitro matured oocytes were vitrified in 5 M ethylene glycol + 5 M propylene glycol with 0.5 M sucrose in TCM-199 + 20% FBS using liquid nitrogen. The highest percentage of morphologically intact in vitro matured oocytes following vitrification was obtained in medium containing 25 ng/mL leptin (89.29%). It was concluded that addition of IGF-I + EGF with 25 ng/mL leptin in MM yielded a higher rate of maturation of oocytes, and supplementation of 25 ng/mL leptin in MM resulted in a higher rate of morphologically intact vitrified oocytes, indicating the efficiency of leptin for cryotolerance.

Key words: Goat, oocytes, in vitro maturation, vitrification, leptin

1. Introduction

Goat is an important livestock species particularly in developing countries because of its ability to utilize different types of forages, and it is very versatile for producers of milk, meat, and skin. Sustained efforts have been made to accelerate genetic gain in goats through the utilization of artificial insemination and multiple ovulation embryo transfer techniques. The culture media employed for in vitro maturation (IVM) not only affect the proportion of mammalian oocytes undergoing fertilization but also influence the subsequent cleavage and developmental competency [1,2]. Varying effects of different additives in culture media were reported for IVM of oocytes. The addition of growth factors, e.g., epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), stimulated oocyte maturation and had beneficial effects on blastocyst production rates in several species [3]. Fetal bovine serum (FBS) is a common supplement for in vitro and ex vivo cell, tissue, and organ cultures [4]. Bovine serum albumin (BSA) improved maturation, fertilization, blastocyst formation, and hatching rates in vitro [5]. Leptin is a 16-kDa peptide hormone secreted mainly by the adipose tissue, which plays an important role in the regulation of food intake, energy expenditure, and reproductive process [6,7]. It was observed that a minimum level of leptin is required for maintenance of reproductive function [8]. Several authors reported expression of leptin in the granular layer of the cumulus oophorus, theca, and interstitial cells of the ovary, testis, and uterus [9,10]. Leptin has also been detected in follicular fluid, granulosa, and cumulus cells in humans and the corpora lutea of rabbits [11]. There was no report available on the supplementation of leptin in maturation medium (MM) for IVM of goat oocytes. Despite several efforts, moderate success has been achieved for the development of blastocysts from in vitro matured and fertilized oocytes. Effective use

* Correspondence: rimtaluk23@gmail.com

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of oocytes in goat reproductive biotechnology requires high quality IVM procedures and effective, reliable cryopreservation techniques. The vitrification technique is a cryoprotectant system involving the addition of higher concentrations of cryoprotectants and ultrarapid cooling, and it has proven to be more effective than slow cooling methods to cryopreserve mammalian oocytes. Effective cryopreservation of in vitro matured oocytes would greatly enhance the availability at the time of need for in vitro production of embryos. It was observed that the supplements used in the medium during IVM of oocytes could influence the rate of in vitro fertilization (IVF) and competence for the development of embryos [12]. It is reported that the cryotolerance of blastocysts after IVF could be affected due to the supplements used in IVM media [13]. Keeping the above in view, the present study was undertaken to find out the effects of certain additives in MM on IVM and vitrification of in vitro matured goat oocytes.

2. Materials and methods

The study and experimental work were conducted at the Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Barapani, Meghalaya, in cooperation with the Department of Animal Reproduction, Gynecology, & Obstetrics, College of Veterinary Science, Assam Agricultural University, Guwahati. The work was undertaken after the approval of the Institutional Animal Ethics Committee.

2.1. Oocyte collection

Ovaries were collected from slaughterhouses in Shillong and bought to the laboratory in thermos flasks maintained at 25–30 °C. After removal of excess tissues from ovaries, oocytes were recovered from the follicles immediately after washing by aspiration technique. The collected oocytes were washed three times in a washing solution containing 5 mg of cysteamine, 3.5 mg of sodium pyruvate, and 100 mL of basic solution; the basic solution contained 20 mL of FBS, 5 mg of gentamicin, and 80 mL of tissue culture medium (TCM-199).

2.2. In vitro maturation of oocytes

The IVM medium was TCM-199 containing 100 µM cysteamine, 5 µg of 17-β estradiol, 5 µg of pure follicle-stimulating hormone (pFSH), and 5 µg of ovine-luteinizing hormone (oLH) per milliliter. Upon IVM with four different concentrations of leptin, namely 10, 20, 25, and 30 ng/mL in IVM medium, 25 ng/mL leptin was found to have increased cumulus expansion and polar body extrusion rate, due to which 25 ng/mL was combined with four different additives, 50 ng/mL IGF-I, 10 ng/mL EGF, 5% FBS, and 2 mg/mL bovine serum albumin (BSA), in maturation medium. The different combinations used were as follows:

M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH
M L: Control + 25 ng/mL leptin
M II: Control + 25 ng/mL leptin + 50 ng/mL IGF-I
M III: Control + 25 ng/mL leptin + 10 ng/mL EGF
M IV: Control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF
M V: Control + 25 ng/mL leptin + 5% FBS
M VI: Control + 25 ng/mL leptin + 2 mg/mL BSA
M VII: Control + 25 ng/mL leptin + 5% FBS + 2 mg/mL BSA

Good-quality oocytes with three to four layers of cumulus cells around the oocytes were included for IVM. Oocytes from the washing medium were transferred to the MM at 4–5 oocytes per droplet and covered with warmed (37–38 °C) sterile mineral oil. The prepared MM was incubated in a CO_2 incubator (5% CO_2) at 38.5 °C with high humidity for 27 h. After completion of maturation, cumulus-oocyte complexes (COCs) were removed by repeated pipetting with 0.1% hyaluronidase and the denuded oocytes were assessed for IVM based on cumulus cell expansion and polar body extrusion under an inverted phase-contrast microscope.

2.3. Vitrification of in vitro matured oocytes

Good-quality in vitro matured oocytes with three to four layers of cumulus cells were used in the present study for vitrification. The cryoprotectant used for vitrification was a combination of 5 M ethylene glycol and 5 M propylene glycol. Leptin (25 ng/mL) in control medium along with the seven different combinations (M I–M VII) of IVM medium were vitrified in cryoprotectants after completion of IVM. First, in vitro matured oocytes were exposed to equilibration solution (ES) containing half the concentration of cryoprotectant with 0.25 M sucrose for about 10 min and then transferred to vitrification solution (VS) with 0.5 M sucrose. Within 30 s, the oocytes with low quantity of VS were loaded in a French medium straw (0.5 mL), which was sealed properly and subjected to vitrification immediately by plunging into a liquid nitrogen (LN_2) container.

2.4. Thawing of vitrified oocytes

Three warming solutions were prepared with the addition of 0.5 M, 0.25 M, and 0.125 M sucrose in the basic solution, respectively. The basic solution contained 80 mL of TCM-199, 20 mL of FBS, and 5.0 mg of gentamicin maintained at a pH of 7.2–7.4. The postthaw vitrified oocytes were examined under a phase-contrast microscope for observing their integrity based on morphology. The oocytes with intact zona pellucida and vitelline membrane, normal spherical shape, and a light and evenly granulated cytoplasm were considered as intact oocytes (Figure 1), whereas oocytes with clear membrane/cytoplasmic damage (brownish, charcoal black cytoplasm and broken

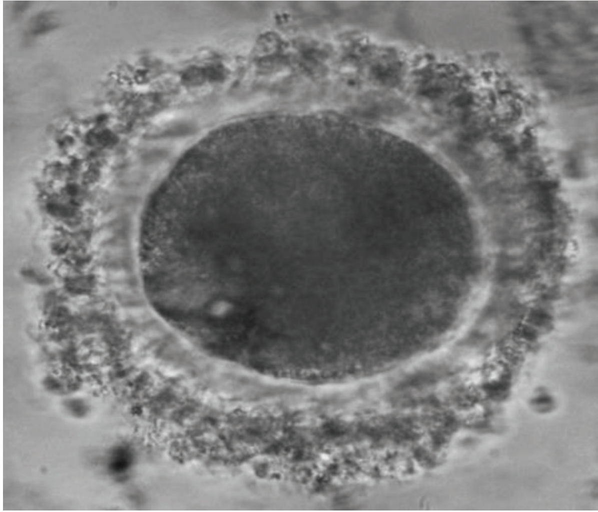

Figure 1. Vitrified matured oocyte with intact cumulus cells under phase-contrast microscope.

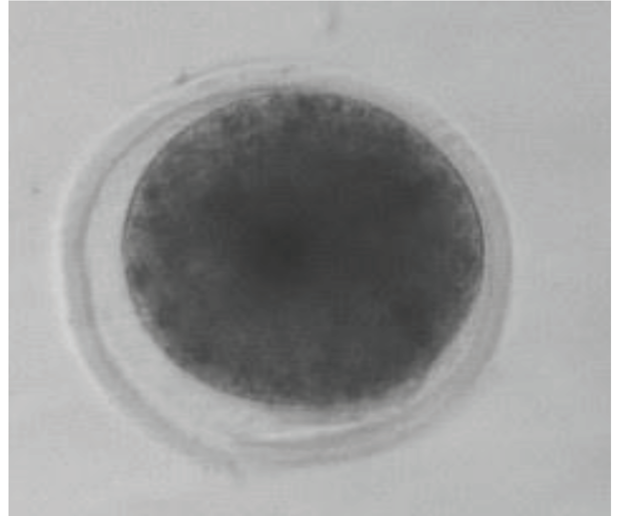

Figure 2. Cracked zona under phase-contrast microscope.

zona pellucida) were considered as damaged oocytes (Figures 2 and 3).

2.5. Statistical analysis

The statistical method used was a chi-square test using SAS Enterprise Guide 4.2.

3. Results

3.1. IVM with different concentrations of leptin

The rates of IVM of oocytes based on cumulus expansion in TCM-199–based medium (Control) with supplementation of 0, 10, 20, 25, and 30 ng/mL leptin, respectively, are provided in Table 1. The rate of IVM obtained with 25 ng/mL leptin was the highest (67.50%) and significantly ($P < 0.01$) higher than that obtained with 10, 20, and 30 ng/mL leptin based on cumulus cell expansion. The rates of IVM of oocytes based on polar body extrusion in TCM-199–based medium did not vary significantly between the concentrations of leptin. The difference in the percentage of oocytes with cumulus expansion differed significantly with 20 ng/mL and 25 ng/mL leptin from that of the control; however, it did not vary significantly with 10 ng/mL and 30 ng/mL leptin from that of control (Table 2).

3.2. IVM in 25 ng/mL leptin with IGF-I, EGF, and combination

The rates of IVM of oocytes based on cumulus expansion and polar body extrusion in different media, M I, M II, M III, and M IV, are presented in Table 3. It was found that medium containing 25 ng/mL leptin in 50 ng/mL IGF-I + 10 ng/mL EGF, i.e. M IV, showed the highest rate of cumulus expansion and polar body extrusion with 66.67% and 28.57%, respectively, which was found to be significantly ($P < 0.05$) higher than that in M I, M II, and M III. The results obtained in independent chi-square tests revealed that the rate of IVM of oocytes based on cumulus expansion in M IV differed significantly ($P < 0.05, 0.01$) from that in M II, M III, and M I (Control). However, the rate of IVM obtained in M II did not differ significantly from that in M III. Also, the rates in M II and M III did not differ significantly from that in M I (Control) (Table 4). Similarly, the results obtained in independent chi-square tests revealed that the rate of IVM of oocytes based on polar body extrusion in M IV differed significantly ($P < 0.05$) from that in M I (Control) but not from M II, M III,

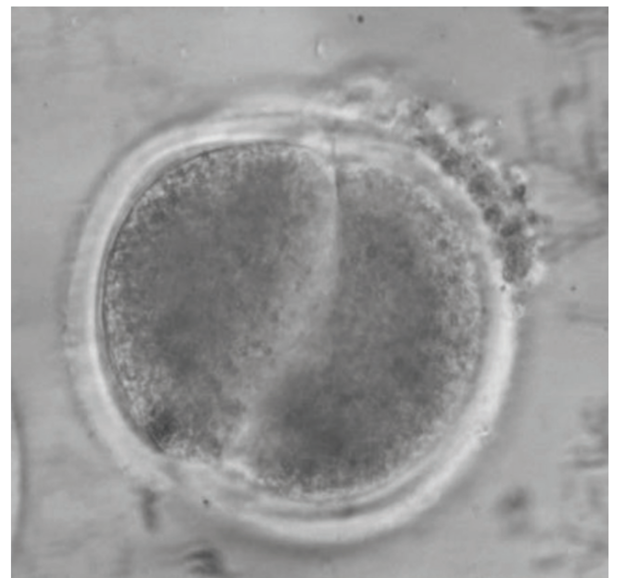

Figure 3. Cracked zona pellucida and split into two halves under phase–contrast. microscope

Table 1. Rate of IVM of goat oocytes based on cumulus cell expansion and polar body extrusion in TCM-199–based medium containing different concentrations of leptin.

Medium	No. of oocytes incubated	Oocytes with cumulus expansion		P value	Oocytes with polar body extrusion		P value
		No.	%		No.	%	
Control (without leptin)	31	11	35.48	172.129** (4.41417E-37)	2	6.45	5.855 ^{NS} (0.210)
Control + 10 ng/mL leptin	32	12	37.50		2	5.00	
Control + 20 ng/mL leptin	38	18	47.37		5	15.63	
Control + 25 ng/mL leptin	40	27	67.50		8	21.05	
Control + 30 ng/mL leptin	34	13	38.24		2	5.88	

**P < 0.01, NS: nonsignificant.

Table 2. Results of independent chi-square tests between IVM media based on cumulus cell expansion in different concentrations of leptin.

Medium	Control (M I)	Control + 10 ng/mL leptin	Control + 20 ng/mL leptin	Control + 25 ng/mL leptin	Control + 30 ng/mL leptin
Control (M I)	--	36.22 ^{NS}	50.02**	51.95**	59.26 ^{NS}
Control + 10 ng/mL leptin	--	--	52.25 ^{NS}	85.09*	40.24 ^{NS}
Control + 20 ng/mL leptin	--	--	--	6.13**	54.43 ^{NS}
Control + 25 ng/mL leptin	--	--	--	--	87.05**
Control + 30 ng/mL leptin	--	--	--	--	--

*P < 0.05, **P < 0.01, NS: nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH.

Table 3. Rate of IVM of goat oocytes based on cumulus cell expansion and polar body extrusion in TCM-199–based medium containing 25 ng/mL leptin with IGF-I, EGF, and combination.

Medium	No. of oocytes incubated	Oocytes with cumulus expansion		P value	Oocytes with polar body extrusion		P value
		No.	%		No	%	
M I (Control)	31	11	35.48	8.755* (0.0327)	2	6.46	8.032* (0.0453)
M II	30	12	40.00		3	10.00	
M III	36	16	44.44		5	13.89	
M IV	42	28	66.67		12	28.57	

*P < 0.05.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M II: control + 25 ng/mL leptin + 50 ng/mL IGF-I; M III: control + 25 ng/mL leptin + 10 ng/mL EGF; M IV: control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF.

and M IV. However, the rate of IVM obtained in M II did not differ significantly from that in M III. Also, the rates in M II and M III did not differ significantly from that in M I (Control) (Table 5).

3.3. IVM in 25 ng/mL leptin with FBS, BSA, and combination

The rates of IVM of oocytes based on cumulus expansion and polar body extrusion in different media, M I, M

Table 4. Results of independent chi-square tests between IVM media based on cumulus cell expansion in TCM-199–based medium containing 25 ng/mL leptin with IGF-I, EGF, and combination.

Medium	Control (M I)	M II	M III	M IV
M I (Control)	--	0.13^{NS}	0.55^{NS}	6.96**
M II	--	--	0.13^{NS}	5.04*
M III	--	--	--	3.89*
M IV	--	--	--	--

*P < 0.05, **P < 0.01, NS: nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M II: control + 25 ng/mL leptin + 50 ng/mL IGF-I; M III: control + 25 ng/mL leptin + 10 ng/mL EGF; M IV: control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF.

Table 5. Results of independent chi-square test between IVM media based on polar body extrusion in TCM-199–based medium containing 25 ng/mL leptin with IGF-I, EGF, and combination.

Medium	Control (M I)	M II	M III	M IV
M I (Control)	--	0.25^{NS}	0.98^{NS}	5.63*
M II	--	--	4.76^{NS}	3.65^{NS}
M III	--	--	--	2.45^{NS}
M IV	--	--	--	--

*P < 0.05, **P < 0.01, NS: nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M II: control + 25 ng/mL leptin + 50 ng/mL IGF-I; M III: control + 25 ng/mL leptin + 10 ng/mL EGF; M IV: control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF.

V, M VI, and M VII, are given in Table 6. Although the rates of IVM did not vary among the media, the medium containing 25 ng/mL leptin with 5% FBS + 2 mg/mL BSA, i.e. M VII, was found to have the highest rates of cumulus expansion and polar body extrusion with 59.62% and 21.15%, respectively.

3.4. Vitrification of in vitro matured oocytes

The percentage of morphologically intact oocytes following maturation in vitro on different media, i.e. M I, M L, M II, M III, and M IV, are presented in Table 7. It was observed that medium containing 25 ng/mL leptin alone, i.e. M L, was found to have a significantly (P < 0.01) higher rate of morphologically intact oocytes with 89.29%, followed by 79.41%, 79.31%, and 75.86% with further addition of 25 ng/mL leptin + IGF-I (M II), 25 ng/mL leptin + IGF-I + EGF (M IV), and 25 ng/mL leptin + EGF (M III), respectively. The rate of morphologically intact oocytes following maturation in control medium (M I) and subsequent vitrification differed significantly (P < 0.01, 0.05) from that in medium with 25 ng/mL leptin (M L), M II, M III, and M IV. However, the rate of morphologically intact oocytes after vitrification did not vary significantly among media with 25 ng/mL leptin (M L), M II, M III, and M IV used for IVM of oocytes in vitro before vitrification (Table 8).

The percentage of morphologically intact oocytes following vitrification on maturation in vitro in different media, i.e. M I, M L, M V, M VI, and M VII, are presented in Table 9. It was observed that medium containing 25 ng/mL leptin alone, i.e. M L, was found to have a significantly (P < 0.01) higher rate of morphologically intact oocytes with 89.29%, followed by 86.49%, 66.67%, and 64.29% with further addition of 25 ng/mL leptin + FBS + BSA (M VII), 25 ng/mL leptin + BSA (M VI), and 25 ng/mL leptin + FBS (M V), respectively. The result of independent chi-square tests revealed that the rate of morphologically intact oocytes following IVM in control medium (M I) and subsequent vitrification differed significantly as compared to the maturation in medium supplemented with 25 ng/mL leptin (M L) and M VII, but not for M V and M VI. The rate of morphologically intact oocytes following maturation in medium containing 25 ng/mL leptin and subsequent vitrification differed significantly (P < 0.05) as compared to the maturation in M V and M VI, but did not differ from M VII. The morphologically intact oocytes matured in M V and subsequent vitrification varied significantly (P < 0.05) from those in matured in M VII but did not differ as compared to maturation in M VI. There was also no significant difference between M VI and M VII (Table 10). This could indicate that maturation of goat oocytes in medium containing 25 ng/mL leptin (M L) was efficacious in preventing cryodamages during the process of vitrification.

4. Discussion

4.1. IVM with different concentrations of leptin

Data on the rate of IVM using leptin in TCM-based medium in goat oocytes are not available in the literature. It was reported that COC expansion, meiotic resumption, and development to the metaphase II (M-II) stage was significantly (P < 0.05) higher in 20 ng/mL leptin in sheep oocytes, whereas the use of 50 ng/mL and 100 ng/mL leptin resulted in lower percentages of oocyte maturation in sheep [14]. Addition of leptin at physiological concentrations (~10 ng/mL) enhanced IVM of adult bovine oocytes as it was found that leptin improved oocyte developmental competence in a dose-dependent manner when matured in the presence of leptin [15,16]. It was also observed that

Table 6. Rate of IVM of goat oocytes based on cumulus cell expansion and polar body extrusion in TCM-199–based medium containing 25 ng/mL leptin with FBS, BSA, and combination.

Medium	No. of oocytes incubated	Oocytes with cumulus expansion		P value	Oocytes with polar body extrusion		P value
		No.	%		No.	%	
M I (Control)	31	11	35.48	4.730^{NS} (0.1920)	2	6.46	3.444^{NS} (0.3281)
M V	45	21	46.67		6	13.33	
M VI	48	23	47.92		8	16.67	
M VII	52	31	59.62		11	21.15	

NS: Nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M V: control + 25 ng/mL leptin + 5% FBS; M VI: control + 25 ng/mL leptin + 2 mg/mL BSA; M VII: control + 25 ng/mL leptin + 5% FBS + 2 mg/mL BSA.

addition of 10 ng/mL leptin in pig oocyte maturation caused significantly higher oocyte maturation rates [17,18]. It was reported that 20 ng/mL leptin enhanced buffalo and camel oocyte maturation rates as well as increasing cleavage and in vitro embryo production [19,20]. Addition of 10 ng/mL leptin to IVM medium improves buffalo oocytes nuclear maturation [21]. It was also seen that addition of 10 ng/mL and 50 ng/mL leptin to IVM medium of buffalo oocytes could increase oocyte nuclear maturation and decrease oocyte apoptosis when trichostatin A was added for inducing apoptosis [22]. It is seen that recombinant human leptin at 10 ng/mL supported the best in vitro growth of sheep preantral follicles (PFs) in terms of growth, increase in diameter, antrum formation, and proportion of oocytes from cultured PFs matured to metaphase II. The inhibitory effect of human leptin on growth of sheep PFs was also observed beyond 25 ng/mL [23]. In the present study, adopting a higher concentration of 30 ng/mL leptin in the medium resulted in a significantly lower percentage of oocytes with cumulus expansion as compared to 25 ng/mL of leptin. It was reported that a high dose of leptin might have an inhibitory effect on ovulation [24], and also on the growth of mouse oocytes, which could be due to blockage of the CAMP pathway [25]. It has been reported that the proportion of PFs exhibiting growth and antrum formation in vitro decreased with increase in leptin concentrations from 10 ng/mL to 50 and 100 ng/mL in goats [26]. In the present study, the proportion of oocytes with cumulus expansion increased with the increase in the concentrations of leptin from 10 to 25 ng/mL. Leptin's influence on reproduction is mediated by the regulation of the hypothalamus-pituitary axis and the ovarian function through its receptor [27,28]. In the ovary, leptin receptor was detected in granulosa cells of follicles, cumulus cells, and oocytes in several species: human, mouse, pig, rabbit, sheep, and cattle [29–32]. It is widely accepted that leptin activates the mitogen-activated protein kinase (MAPK) pathway, leading to the induction of cellular maturation. Interaction between leptin and cumulus cell-enclosed oocytes could modulate steroid production in cumulus cells that control oocyte physiology by secreting steroids [33,34]. In some species, leptin seems to promote rearrangement of cytoskeletal elements that are involved in chromosome segregation and organelle movement [35,36]. Other studies reported that leptin enhanced an appropriate spindle assembly during metaphase and stimulated meiotic oocyte maturation in mouse, pig, and cattle [37–40].

Table 7. Rate of morphologically intact goat oocytes following vitrification on MM in vitro in TCM-199 along with leptin, IGF-I, EGF, and their combination.

Medium	No. of oocytes matured and vitrified	Intact oocytes		P value
		No.	%	
M I (Control)	20	9	45.00	13.523** (0.0090)
M L	28	25	89.29	
M II	34	27	79.41	
M III	29	22	75.86	
M IV	29	23	79.31	

**$P < 0.01$.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M L: control + 25 ng/mL leptin; M II: control + 25 ng/mL leptin + 50 ng/mL IGF-I; M III: control + 25 ng/mL leptin + 10 ng/mL EGF; M IV: control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF.

4.2. IVM in 25 ng/mL leptin with different combinations of IGF-I, EGF, FBS, and BSA

Reports on effects of supplementation of growth factors, serum, and leptin in the MM on the comparative efficacy of IVM were not available in the literature. In the present

Table 8. Independent chi-square tests of morphologically intact oocytes following maturation in different media and subsequent vitrification.

Medium	M I (Control)	Control + 25 ng/mL Leptin	M II	M III	M IV
M I (Control)	--	11.07**	6.71**	4.85*	6.15*
M L	--	--	1.10 NS	1.77 NS	1.06 NS
M II	--	--	--	0.11 NS	0.99NS
M III	--	--	--	--	--
M IV	--	--	--	--	--

*P < 0.05, **P < 0.01, NS: nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M L: control + 25 ng/mL leptin; M II: control + 25 ng/mL leptin + 50 ng/mL IGF-I; M III: control + 25 ng/mL leptin + 10 ng/mL EGF; M IV: control + 25 ng/mL leptin + 50 ng/mL IGF-I + 10 ng/mL EGF.

Table 9. Rate of morphologically intact goat oocytes following vitrification on MM in vitro in TCM-199 along with leptin, FBS, BSA, and their combination.

Medium	No. of oocytes matured and vitrified	Intact oocytes		P value
		No.	%	
M I (Control)	20	9	45.00	16.701** (0.0022)
M L	28	25	89.29	
M V	28	18	64.29	
M VI	24	16	66.67	
M VII	37	32	86.49	

**P < 0.01.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M L: control + 25 ng/mL leptin; M V: control + 25 ng/mL leptin + 5% FBS; M VI: control + 25 ng/mL leptin + 2 mg/mL BSA; M VII: control + 25 ng/mL leptin + 5% FBS + 2 mg/mL BSA.

study, different combinations of additives were added to MM consisting of Tris-based control medium along with 25 ng/mL leptin to find their efficiency on IVM. It was found that the percentage of cumulus expansion of oocytes was significantly higher in TCM-199 with leptin + IGF-I +EGF as compared to leptin + IGF-I and leptin + EGF. Based on polar body extrusion, leptin + IGF-I + EGF had a higher percentage as compared to the others although this did not vary significantly. The findings were indicative of the superiority of the combination of IGF and EGF on IVM of oocytes. IGF-I is known to stimulate protein synthesis when added to the medium for mouse embryos in vitro and increase estradiol production by the theca granulosa cells in serum-free culture [41,42]. It was reported that the biological effects of IGF-I are mediated by its interaction with the IGF type I receptor and modulated by IGF binding proteins [43]. An increase in the level of IGF binding protein may alter the bioavailability of IGF, thus stimulating steroidogenesis and mitogenesis in developing follicles [44]. The growth factors acting in the presence of cumulus cells transfer a positive signal for oocytes maturation, possibly by the synthesis of new proteins. It was observed that the combined effect of EGF and IGF-I appeared to be mediated by the surrounding cumulus cells [45]. It has been observed that IGFs have an affinity for soluble binding proteins that can modulate receptor binding and hence influence their biological activity [46]. It was found that the cleavage rate and embryo development to the 8- to 16-cell stage in bovine embryos were higher in 100 ng/mL IGF-I and in 5 ng/mL leptin + 100 ng/mL IGF-I at 87% and 51%, respectively. The proportions of embryos reaching the blastocyst stage on day 8 were 26.7%, 29.6%, 31.5%, and 29.8% for the control, 5 ng/mL leptin, 100 ng/mL IGF-I, and 5 ng/mL leptin + 100 ng/mL IGF-I groups, respectively [47].

EGF might act on the cumulus cells and/or on the oocyte itself since mRNA for the EGF receptor was stated to be present in the bovine oocyte, which could be extrapolated to goat oocytes [48]. It was reported that the mechanism by which growth factors regulate or modulate resumption of meiosis in oocytes might be modulated via the granulose and/or cumulus cells [49]. It was found that the goat cumulus cells express the EGF receptor and EGF, being a polypeptide with potent mitogenic activity, was reported to trigger signaling through the mitogen-activated protein kinase pathway [50]. The factors secreted by the cumulus cells regulate the disruption of the gap junction and cumulus expansion in pig oocytes [51].

Table 10. Independent chi-square tests of morphologically intact oocytes following maturation in different media and subsequent vitrification.

Medium	M I (Control)	Control + 25 ng/mL leptin	M V	M VI	M VII
M I (Control)	--	11.07**	1.76 NS	0.14 NS	11.06**
M L	--	--	4.90*	3.96*	0.111 NS
M V	--	--	--	0.03NS	4.42*
M VI	--	--	--	--	3.41 NS
M VII	--	--	--	--	--

*P < 0.05, **P < 0.01, NS: nonsignificant.
M I/Control: TCM-199 + 100 µM cysteamine + 5 µg 17-β estradiol + 5 µg pFSH + 5 µg oLH; M L: control + 25 ng/mL leptin, M V: control + 25 ng/mL leptin + 5% FBS; M VI: control + 25 ng/mL leptin + 2 mg/mL BSA; M VII: control + 25 ng/mL leptin + 5% FBS + 2 mg/mL BSA.

Other studies reported that oocytes obtained in medium supplemented with growth hormone (GH), IGF-I, EGF, thyroxin (T4), and FSH had the maximum percentage of M-II stage oocytes (38.6%) when compared to the other treatments, revealing that in vitro development of goat PFs could be significantly improved through addition of GH, EGF, T4, and FSH in the best combination [52].

However, differential rates of maturation of cattle oocytes in vitro were found upon supplementation of FBS (81.7% ± 12.9%) and estrous calf serum (85.7 ± 12.7%) in TCM-199 containing 5 µg/mL FSH and 1 µg/mL estradiol [53].

4.3. Vitrification of in vitro matured oocytes

The cryopreservation of mature oocytes is known to induce disruption of the spindle, chromosomes, microfilaments, and cortical granule distribution. Addition of leptin in MM might have helped in reducing the damages induced during the process of vitrification since leptin appeared to promote rearrangement of cytoskeletal elements that are involved in chromosome segregation and organelle movement. However, vitrification of IGF-I, EGF, and IGF-I + EGF along with leptin added to MM did not improve the rate of intact oocytes following vitrification of matured oocytes. This could be due to the inhibitory effect of leptin used in combination with growth factors and hormones [54]. It was reported that inclusion of human or ovine leptin (10 ng/mL) along with FSH, T4, IGF-I, and GH generated only marginal improvements during in vitro development of sheep preantral follicles. The percentage of morphologically intact vitrified oocytes following maturation in medium containing 25 ng/mL leptin was significantly (P < 0.01) higher than that with leptin + 5% FBS and leptin + 2 mg/mL BSA. This could be due to the influence of the combined sera in conjugation with leptin that could cause a higher level of intact vitrified oocytes since serum is known to influence cryotolerance. The use of a combination of sera during IVM was also found to bring about a significantly higher rate of intact vitrified oocytes after maturation compared to a single serum (5% FBS) in the present investigation, which could substantiate the beneficial effects of combined sera.

5. Conclusion

The present findings signify the importance of leptin supplementation during IVM of goat oocytes. A higher percentage of morphologically intact in vitro matured vitrified oocytes was observed in medium supplemented with 25 ng/mL leptin, which shows its significance in maturation and vitrification of goat oocytes.

Acknowledgments

We are grateful to the Director of the Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Umiam, Meghalaya, and the Head of the Animal Production Division for providing the infrastructure required during the period of study.

Conflict of interest

None of the authors have a conflict of interest to declare.

Funding organization

The funds were provided in the form of a student's fellowship by the Department of Biotechnology, Government of India.

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