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

Reema TALUKDAR1*, Ranjan Kumar BISWAS1, Govindasamy KADIRVEL2, Bharat Chandra DEKA2, Sudip SINHA2, Lukumoni BURAGOHAIN2, Birina BORA4
1Department of Animal Reproduction, Gynecology, & Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, India
2Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Umiam, Meghalaya, India
3Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, India
4Department of Mathematical Science, University of Delhi, Delhi, India

* Correspondence: rmtaluk23@gmail.com

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
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 brought 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 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.

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 II**: Control + 25 ng/mL leptin
- **M III**: Control + 25 ng/mL leptin + 50 ng/mL IGF-I
- **M IV**: Control + 25 ng/mL leptin + 10 ng/mL EGF
- **M V**: Control + 25 ng/mL leptin + 25 ng/mL leptin + 5% FBS + 2 mg/mL BSA
- **M VI**: Control + 25 ng/mL leptin + 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 CO₂ incubator (5% CO₂) 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 oocytes 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) 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
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 1. Vitrified matured oocyte with intact cumulus cells under phase-contrast microscope.

Figure 2. Cracked zona under phase-contrast microscope.

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**                     | 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 | Oocytes with polar body extrusion | P value          | 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 | 0.55  | 6.96 |
| M II          | --            | --   | 0.13  | 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 | 0.98  | 5.63 |
| M II          | --            | --   | 4.76  | 3.65 |
| M III         | --            | --   | --    | 2.45 |
| 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 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 < 0.05) from that in medium 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 medium supplemented with 25 ng/mL leptin (M L) and M VII, but did not differ from M V and M VI. The rate of 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...
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].

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

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<sup>NS</sup> (0.1920) | 2     | 6.46                  | 3.444<sup>NS</sup> (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.

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.
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 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    | --           | 11.07**                   | 6.71* | 4.85* | 6.15* |
| M L    | --           | --                        | 1.10 NS | 1.77 NS | 1.06 NS |
| M II   | --           | --                        | 0.11 NS | 0.99 NS |
| 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 FSH + 5 µg LH; 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 FSH + 5 µg LH; 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 conjunction 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.

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 | -- | -- | -- | 0.03 NS | 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.
References

1. Tajik P, Esfandabadi NS. In vitro maturation of caprine oocytes in different culture media. Small Ruminant Research 2003; 47 (2): 155-158. doi: 10.1016/S0921-4488(02)00238-9

2. Madan ML, Chauhan MS, Singla SK, Manik RS. Pregnancies established from water buffalo (Bubalus bubalis) blastocysts derived from in vitro matured, in vitro fertilized and co-cultured with cumulus and oviductal cells. Theriogenology 1994; 42 (4): 591-600. doi: 10.1016/0093-691x(94)90376-1

3. Harper KM, Bracket BG. Bovine blastocyst development after in vitro maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. Biology of Reproduction 1993; 48 (2): 409-416. doi: 10.1095/biolreprod48.2.409

4. Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F et al. The human collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicology In Vitro 2004; 18 (1): 1-12. doi: 10.1016/j.tiv.2003.08.009

5. Visconti PE, Bailey JL, Moore GO, Pan D, Olds-Clarke P et al. Capacitation of mouse spermatozoa part I: Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995; 121 (4): 1129-1137.

6. Keim NL, Stern JS, Havel PJ. Relation between circulating leptin concentrations and appetite during a prolonged, moderate energy deficit in women. American Journal of Clinical Nutrition 1998; 68 (4): 794-801. doi: 10.1093/ajcn/68.4.794

7. Cervero A, Horcajadas JA, Martin J, Pellicer A, Simon C. The leptin system during human endometrial receptivity and preimplantation. Journal of Clinical Endocrinology and Metabolism 2004; 89 (5): 2442-2451. doi: 10.1210/jc.2003-032127

8. Cheung CC, Thornton JE, Kuijper JL, Weigle DS, Clifton DK et al. Leptin is a metabolic gate for the onset of puberty in the female rat. Endocrinology 1997; 138 (2): 855-858. doi: 10.1210/endo.138.2.5054

9. Cioffi JA, Van Blerkom J, Antczak M, Schafer A, Wittmer S et al. The expression of leptin and its receptors in preovulatory human follicles. Molecular Human Reproduction 1997; 3 (6): 467-472. doi: 10.1093/molehr/3.6.467

10. Karlsson C, Lindell K, Svensson E, Bergh C, Lind P. Expression of functional leptin receptors in the human ovary. Journal of Clinical Endocrinology and Metabolism 1997; 82 (12): 4144-4148. doi: 10.1210/jcem.82.12.4446

11. Zerani M, Boiti C, Zampini D, Brecchia G, Dall’Aglio C et al. Ob receptor in rabbit ovary and leptin in vitro regulation of corpora lutea. Journal of Endocrinology 2004; 183 (2): 279-288. doi: 10.1677/joe.1.05507

12. Ali A, Sirard MA. Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in vitro maturation. Biology of Reproduction 2002; 66 (4): 901-905. doi: 10.1095/biolreprod66.4.901

13. Shirazi A, Ansari Ardali M, Ahmadi E, Nazari H, Mamuee M et al. The effect of macromolecule source and type of media during in vitro maturation of sheep oocytes on subsequent embryo development. Journal of Reproduction and Fertility 2012; 13 (1): 13-19.

14. Keshrawani S, Kumari GA, Reddy KRC. Supplementation of leptin on in vitro maturation of sheep oocytes. Asian Journal of Animal and Veterinary Advances 2016; 11 (10): 629-636. doi: 10.3923/aja.2016.629.636

15. Boelhauve MF, Sinowatz F, Wolf E, Paula-Lopes FF. Maturation of bovine oocytes in the presence of leptin improves development and reduces apoptosis of in vitro-produced blastocysts. Biology of Reproduction 2005; 73 (4): 737-744. doi: 10.1095/biolreprod.105.041103

16. Paula-Lopes FF, Boelhauve M, Habermann F, Sinowatz F, Wolf E. Differential mechanism of leptin action improving nuclear maturation and developmental competence of bovine oocytes. Reproduction, Fertility and Development 2005; 18 (2): 277-277. doi: 10.1071/RDv18n2Ab339

17. Cord J, Zhu H, Dyce PW, Petrlik J, Li J. Leptin enhances oocytes nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway. Endocrinology 2004; 145 (11): 5355-5363. doi: 10.1210/en.2004-0783

18. Kun Z, Shaohua W, Yufang M, Yankun L, Hengxi W et al. Effects of leptin supplementation in in vitro maturation medium on meiotic maturation of oocytes and preimplantation development of parthenogenetic and cloned embryos in pigs. Animal Reproduction Science 2007; 101 (1-2): 85-96. doi: 10.1016/j.anireprosci.2006.08.021

19. Singh B, Prasad S, Gupta HP. Effect of leptin on in vitro maturation of oocytes and on early embryonic development in buffaloes. Indian Journal of Animal Reproduction 2012; 33 (2): 1-6.

20. Gabr SA, Samia SM, Nagy WM. Effect of leptin supplementation in maturation medium on in vitro nuclear maturation and fertilization of camel oocytes (Camelus dromedaries). Asian Journal of Animal and Veterinary Advances 2014; 9 (11): 713-720. doi: 10.3923/aja.2014.713.720

21. Khaki A, Batavani R, Najafi GR, Tahmasbian H, Belbasi A et al. Effect of leptin on in vitro nuclear maturation and apoptosis of buffalo (Bubalus bubalis) oocyte. International Journal of Fertility & Sterility 2014; 8 (1): 43-50.

22. Sheykhani HRS, Batavani RA, Najafi GR. Protective effect of leptin on induced apoptosis with trichostatin A on buffalo oocytes. Veterinary Research Forum 2016; 7 (2): 99-104.

23. Kamalamma P, Kona SSR, Praveen Chakravarthi V, Siva Kumar AVN, Punyakumari B et al. Effect of leptin on in vitro development of ovine preantral ovarian follicles. Theriogenology 2015; 85 (2): 224-229. doi: 10.1016/j.theriogenology.2015.09.045

24. Duggal PS, Weitsman SR, Magoffin DA, Norman RJ. Expression of the long (OB-RB) and short (OB-RA) forms of the leptin receptor throughout the oestrous cycle in the mature rat ovary. Reproduction 2002; 123 (6): 899-905.
25. Swain JE, Dunn RL, McConnel D, Gonzalez-Martinez J, Smith GD. Direct effects of leptin on mouse reproductive function: regulation of follicular oocytes and embryo development. *Biology of Reproduction* 2004; 71 (5): 1446-1452. doi: 10.1095/biolreprod.104.033035

26. Divya V, Arunakumari G, Sadasiva Rao K. Effect of leptin on in vitro development of caprine preantral follicles. *International Journal of Agricultural Science* 2018; 10 (6): 5481-5484.

27. Chehab FF, Li ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics* 1996; 12 (3): 318-320. doi: 10.1038/ng0396-318

28. Brecchia G, Bonanno A, Galeati G, Federici G, Maranesi M et al. Hormonal and metabolic adaptation to fasting: effects on the hypothalamic–pituitary–ovarian axis and reproductive performance of rabbit does. *Domestic Animal Endocrinology* 2006; 31 (2): 105-122. doi: 10.1016/j.domianend.2005.09.006

29. Antczak M, Van Blerkom J. Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Molecular Human Reproduction* 1997; 3 (12): 1067-1086. doi: 10.1093/molehr/3.12.1067

30. Ryan NK, Woodhouse CM, Van der Hoek KH, Gilchrist RB, Armstrong DT et al. Expression of leptin and its receptor in the murine ovary: possible role in the regulation of oocytes maturation. *Biology of Reproduction* 2002; 66 (5): 1548-1554. doi: 10.1095/biolreprod66.5.1548

31. Muñoz-Gutiérrez M, Findlay PA, Adam CL, Wax G, Campbell BK et al. The ovarian expression of mRNAs for aromatase, IGF-1 receptor, IGF binding protein-2, -4 and -5, leptin and leptin receptor in cycling ewes after three days of leptin infusion. *Reproduction* 2005; 130: 869-881. doi: 10.1530/rep.1.00557

32. Paula-Lopes FF, Boelhaue M, Habermann FA, Sinowatz F, Wolf E. Leptin promotes meiotic progression and developmental capacity of bovine oocytes via cumulus cell independent and dependent mechanisms. *Biology of Reproduction* 2007; 76 (3): 532-541. doi: 10.1095/biolreprod.106.054551

33. Karamouti M, Kollia P, Karligiotou E, Kallitsaris A, Prapas N et al. Absence of leptin expression and secretion by human luteinized granulosa cells. *Journal of Molecular Endocrinology* 2003; 31 (1): 233-239. doi: 0952-5041/03/031-233

34. Mingotti GZ, Garcia JM, Rosa-e-Silva AAM. The effect of serum on in vitro maturation, in vitro fertilization and steroidogenesis of bovine oocytes co-cultured with granulose cells. *Brazilian Journal of Medical and Biological Research* 1995; 28 (2): 213-217.

35. Suzuki H, Sasaki Y, Shimizu M, Matsuzaki M, Hashizume T et al. Ghrelin and leptin did not improve meiotic maturation of porcine oocytes cultured in vitro. *Reproduction in Domestic Animals* 2010; 45 (5): 927-930. doi: 10.1111/j.1439-0531.2009.01352.x

36. Sun QY, Schatten H. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction* 2006; 131 (2): 193-205. doi: 10.1530/rep.1.00847

37. Younis AI, Brackett BG, Fayrer-Hosken RA. Influence of serum and hormone on bovine oocyte maturation and fertilization in vitro. *Gamete Research* 1989; 23 (2): 189-201. doi: 10.1002/mrd.1120230206

38. Matsuoka T, Tahara M, Yokoi T, Masumoto N, Takeda T et al. Tyrosine phosphorylation of STAT3 by leptin through leptin receptor in mouse metaphase 2 stage oocyte. Biochemical and Biophysical Research Communications 1999; 256 (3): 480-484. doi: 10.1006/wwcc.1999.0365

39. Kun Z, Shaohua W, Yufang M, Hankun L, Mengxi W et al. Effects of leptin supplementation in in vitro maturation medium on meiotic maturation of oocytes and preimplantation development of parthenogenetic and cloned embryos in pigs. *Animal Reproduction Science* 2007; 101 (1-2): 85-96. doi: org/10.1016/j.anireprosci.2006.08.021

40. Van Tol HTA, Van Eerdenburg FJCM, Colenbrander B, Roelen BAJ. Enhancement of Bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. *Molecular Reproduction and Development* 2008; 75 (4): 578-587. doi: 10.1002/mrd.20801

41. Simmen RCM, Ko Y, Simmen FA. Insulin-like growth factors and blastocyst development. *Theriogenology* 1993; 39 (1): 163-175. doi: 10.1016/0093-691X(93)90031-Y

42. Shores EM, Picton HM, Hunter MG. Differential regulation of pig theca cell steroidogenesis by LH, insulin-like growth factor I and granulosal cells in serum-free culture. *Journal of Reproduction and Fertility* 2000; 118 (2): 211-219. doi: 10.1530/jrf.0.1180211

43. Jones JJ, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* 1995; 16 (1): 3-34. doi: 10.1210/edrv-16-1-3

44. Bridges TS, Davidson TR, Chamberlain CS, Geisert RD, Spicer LJ. Changes in follicular fluid steroids, insulin-like growth factors (IGF) and IGF-binding protein concentration, and proteolytic activity during equine follicular development. *Journal of Animal Science* 2002; 80 (1): 179-190. doi: 10.2527/2002.801179

45. Lorenzo PL, Illera MJ, Illera JC, Illera M. Enhancement of cumulus expansion and nuclear maturation during bovine oocytes maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor-I. *Journal of Reproduction and Fertility* 1994; 101 (3): 697-701. doi: 10.1530/jrf.0.1010697

46. Palma GA, Müller M, Brem G. Effect of insulin-like growth factor 1 (IGF-I) at high concentrations on blastocyst development of bovine embryos produced in vitro. *Journal of Reproduction and Fertility* 1997; 110 (2): 347-353. doi: 10.1530/jrf.0.1100347

47. Kaya A, Sağirkaya H, Misirlioğlu M, Gümen A, Parrish JJ et al. Leptin and IGF-I improve bovine embryo quality in vitro. *Animal Reproduction* 2017; 14 (4): 1151-1160. doi: 10.21451/1984-3143-AR987
48. Banwell KM, Thompson JG. In-vitro maturation of mammalian oocytes: Outcomes and consequences. Seminars in Reproductive Medicine 2008; 26 (2): 162-174. doi: 10.1055/s-2008-1042955

49. Brucker C, Alexander NJ, Hodgen GD, Sandow BA. Transforming growth factor-alpha augments meiotic maturation of cumulus cell-enclosed mouse oocytes. Molecular Reproduction & Development 1991; 28 (1): 94-98. doi: 10.1002/mrd.1080280115

50. Gall L, Chene N, Dahirel M, Ruffini S, Boulesteix C. Expression of epidermal growth factor receptor in the goat cumulus-oocyte complex. Molecular Reproduction & Development 2004; 67 (4): 439-445. doi: 10.1002/mrd.20040

51. Isobe N, Terada T. Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus-oocyte complexes cultured in-vitro. Reproduction 2001; 121 (2): 249-257. doi: 10.1530/reprod/121.2.249

52. Shankaraiah P, Swathi B, Kumari GA, Priyanka, Prasad CS et al. Effect of different combinations of the growth factors and hormones on in vitro maturation of goat preantral follicles. International Journal of Current Microbiology and Applied Sciences 2018; 7 (4): 1956-1963. doi: 10.20546/ijcmas.2018.704.224

53. Singha JK, Bhuian MKU, Rahman MM, Bari FY. Comparison of culture media for in vitro maturation of oocytes indigenous Zebu cows in Bangladesh. Journal of Embryo Transfer 2015; 30 (4): 327-333. doi: 10.12750/JET.2015.30.4.327

54. Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP et al. The stomach is a source of leptin. Nature 1998; 394 (6695): 790-793. doi: 10.1038/29547