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Girin Hazarika
Ph.D., Scholar, Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Ranjan Kumar Biswas
Professor, Department of Animal Reproduction Gynaecology & Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Prabodh Borah
Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Devojyoti Dutta
Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Nikhil Chandra Nath
Assistant Professor, Department of Veterinary Physiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Manoj Kumar Kalita
Ph.D., Scholar, Department of Animal Reproduction Gynaecology & Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Bharat Chandra Deka
Retd Professor, Department of Animal Reproduction Gynaecology & Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Correspondence
Girin Hazarika
Ph.D., Scholar, Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India

Effect of hormone combination on *in vitro* maturation of goat oocytes and its diametrical evaluation of expansion in different IVM media

Girin Hazarika, Ranjan Kumar Biswas, Prabodh Borah, Devojyoti Dutta, Nikhil Chandra Nath, Manoj Kumar Kalita and Bharat Chandra Deka

Abstract
Diametrical measurement of expansion of goat oocytes (COCs) after IVM for 24 hrs, 38.5^{0}C, 5% CO2 was evaluated objectively in different media, *viz*., 1 to 4. The maturation based on cumulus cells expansion was highest (84.24%) in medium 4. The mean diameter (µm) of oocytes with cumulus cells before and after IVM varied significantly (P<0.05) in all media for all types of oocytes, except for C type of oocytes in medium 1. The increment in the mean diameter of oocytes with cumulus cells after IVM differed significantly (P<0.05) between all the media for B and C types oocyte. The increase in the mean diameter of COCs was significantly (P<0.05) higher in medium 4 for all types of oocytes. Increase mean diameter between all types oocyte with cumulus cells after IVM differed significantly (P<0.05) in medium 1. In medium 2 the values for C type of oocytes was significantly (P<0.05) lower than that of A and B types oocytes. The increment in mean diameter for A type oocytes was significantly (P<0.05) higher from B and C types oocytes in medium 3. In medium 4, increase in mean diameter of A type of oocytes differed significantly (P<0.05) from C type oocytes but not from B type oocytes and varied significantly between B and C types oocyte mean diameter.

Keywords: Hormone combination, maturation diametrical evaluation, IVM media

Introduction
Application of Assisted Reproductive Technology like multiple ovulation and embryo transfer (MOET) is aimed towards increasing reproductive efficiency and accelerating genetic gain. MOET increases the number of offsprings from genetically superior females. However, variable response to hormonal treatment, fertilization failures and premature regression of corpora lutea limit the applicability of MOET which became a major constraint in large scale adoption of embryo transfer programme. Surgical intervention is required in goat for harvesting embryos following superovulation which is costly and cumbersome and restricts repeated applications. *In vitro* maturation (IVM) of oocytes and *in vitro* fertilization (IVF) offer a viable alternative to MOET for production of large number of viable embryos. The success rate of IVM depends largely on selection of media and additives (Staigmiller *et al.* 1984) [13] and (Rose *et al*. 1992) [13, 11] and environment of culture of oocytes. Ocana-quero *et al.* (1999) [9] and Zi *et al.* (2009) [9, 19]. Scanty investigation on IVM of goat oocytes in different culture media supplemented with hormones and other chemicals has been carried out in India Chauhan and Anand (1991) [4], Pawshe *et al.* (1993) [10] and Biswas (1998) [4, 10, 2].
Expansion of cumulus-oocytes-complexes (COCs) during IVM is indicative of cytoplasmic maturation of oocytes in a medium which is recorded subjectively under microscope as a component of oocyte maturation. Objective evaluation of the extent of expansion of COCs after IVM by means of physical measurement has so far remained unattempted. Therefore an effort has been made in the present study to evaluate the enlargement of goat COCs objectively by recording the diameter of COCs of different types of oocyte under the microscope before and after IVM in different media which was reflective of extent of cumulus cells expansion indicating efficacy of media for cytoplasmic maturation of oocytes.

Materials and Methods
Ovaries of adult goats were collected from the local abattoirs of Guwahati (Assam) as soon as possible after the goats were slaughtered. A total of 185 ovaries were obtained for the study.

Ovaries were carried to the laboratory in a thermos flask containing warm (37ºC) normal saline solution (NSS) with antibiotics and washed 3-4 times with NSS containing Gentamicin (Cat.No. G1272, Sigma-Aldrich) prior to further processing. A total of 674 oocytes were recovered from the ovaries immediately after wash by slicing technique. Oocytes recovered from the ovaries were searched out under a steriozoom microscope (Nikon SMZ 1000, Model CDSS230, Japan) and collected with the help of a lifter. The oocytes with cumulus cells *i.e.*, COCs were then washed 5-6 times in a washing medium which contained 36 ml TCM-199 (Cat.No. M7528, Sigma-Aldrich), 4 ml Foetal bovine serum (Cat.No.RM9955, Sigma-Aldrich), 0.0036g Sodium pyruvate (Cat.No.P4562, Sigma-Aldrich), 0.004 g L-glutamine (Cat.No.G8540, Sigma-Aldrich) and 200µl Gentamicin (Cat. No. G1272, Sigma-Aldrich) in a volume of 40 ml. After thorough mixing of the ingredients, the washing medium was filtered using 0.22µm syringe filter (Cat No. MG25020NYSL, Genetix) and then kept in a CO_2 incubator (Galaxy 48S, Eppendorf, Germany) maintaining 5 per cent CO_2 at 38.5ºC with 90-95 per cent relative humidity for 2 hours, prior to use. Following washing, oocytes were classified into three types (A, B and C) based on their gross morphology and integrity of cumulus cells, Type A: Oocytes surrounded by 3 or more complete layers of cumulus cells adhered to the zona pellucida, Type B: Oocytes surrounded by 2 complete layers of cumulus cells adhered to the zona pellucida and Type C: Oocytes surrounded by 1 complete layer of cumulus cells adhered to the zona pellucida. Oocytes having less than 1 complete layer were not used in the study.

IVM of oocytes

Preparation of culture medium for IVM of oocytes

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Four different IVM media were prepared using TCM 199 as base medium. Compositions of the IVM media used were as follows:

Medium 1(10ml) (Control): Washing medium (9ml) + cysteamine (100µM/ml) (Cat.No.M9768, Sigma-Aldrich) + 17β oestradiol (1µg/ml) (Cat. No. E-2758, Sigma-Aldrich) + pFSH (5µg/ml) (Cat. No. F-2293, Sigma-Aldrich). Medium 2(10ml): Washing medium (9ml) + cysteamine (100µM/ml) + 17β oestradiol (1µg/ml) + pFSH (5µg/ml)+ Heat-inactivated kid serum (1ml). Medium 3 (10ml): Washing medium (9ml) + cysteamine (100µM/ml) + 17β oestradiol (1µg/ml) + Heat-inactivated kid serum (1ml). Medium 4 (10ml): Washing medium (9ml) + cysteamine (100µM/ml) + 17β oestradiol (1µg/ml) + pFSH (5µg/ml) + hLH (5µg/ml) (Cat.No. L6420, Sigma-Aldrich).

The media were incubated for 2 hours in 5 per cent CO_2 at 38.5ºC with 90-95 per cent humidity and were filtered through 0.22µm filter just before use.

Incubation of oocytes

With the help of a micropipette, 50µl of maturation medium (as IVM droplet) was placed gently on a 35mm petri dish (Cat No.150318, Nunc). Different types of oocytes were transferred into the maturation media @10-12 oocytes / droplet and covered with warm (37ºC-38ºC) mineral oil. The petri dish was then placed in a CO_2 incubator maintaining 5 per cent CO_2 and 90-95 per cent relative humidity for 24 hours at a temperature of 38.5ºC.

Assessment of cumulus expansion of oocytes subjected to IVM

After 24 hours of incubation in IVM media, the degree of cumulus expansion of different types of oocyte was determined microscopically (Figure 2) for which the measurement of oocyte diameter with cumulus cells was recorded before and after IVM under a phase-contrast microscope (Nikon, Model: DMIL LED). Images of COCs were captured at 200X magnification and the measurements for different types of oocyte in different maturation media were taken by using Leica Application suite EZ V1.8.4 software connected with the microscope.

Statistical Analysis

Data obtained in the present experiment were analyzed statistically by SAS Enterprise Guide 4.3.

Results

IVM of Oocytes

The IVM performance of oocytes on the basis of cumulus cell expansion in different media is presented as percentage of maturation in Figure 1. Chi-square test revealed that there was no significant difference in the percentage of oocyte maturation between different maturation media.

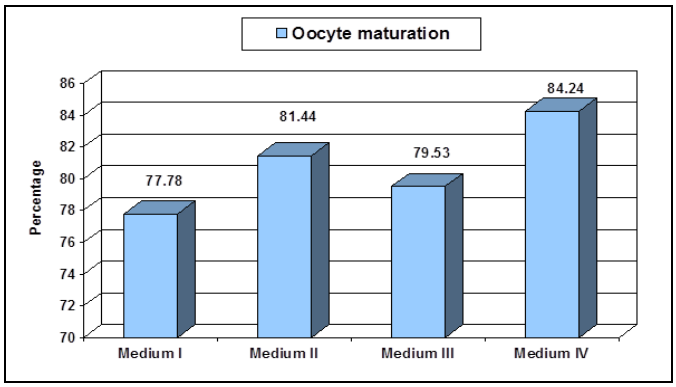

Fig 1: Percentage of *in vitro* matured oocytes based on cumulus cell expansion in different media.

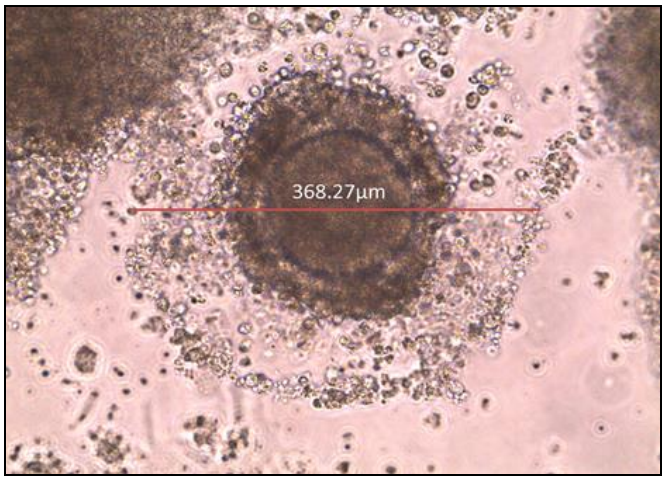

Fig 2: Diameter of oocyte after maturation

Assessment of cumulus expansion of oocytes subjected to IVM

The measurements of diameter of oocytes with intact cumulus cells before maturation and that of oocytes with expanded

cumulus cells after IVM in different media for different types of oocyte are presented in Table1. Analysis of variance revealed that the mean diameter of the oocytes with cumulus cells before and after IVM differed significantly (P<0.05) in different media. It was observed that the mean diameter of the oocytes with cumulus cells varied significantly (P<0.05) before and after IVM in all the media for A and B types of oocyte. It also varied significantly (P<0.05) between before and after IVM in media 2, 3 and 4 but not in medium 1(Control) for C type of oocyte (Table1). The increase in mean diameter of oocytes with cumulus cells after IVM in different media for A, B and C types of oocyte is furnished in Table 2. Analysis of variance showed that the increase in mean diameter of the oocytes with cumulus cells differed significantly (P<0.05) between the media for all the types of oocytes. The values also varied significantly (P<0.05) between the types of oocyte within the media.

Table 1: Diameter (µm) of oocytes with cumulus cells (mean±se) before and after *in vitro* maturation in different media

Media	Type of oocyte					
	A		B		C	
	Before maturation	After maturation	Before maturation	After maturation	Before maturation	After maturation
Medium 1	368.56^{b}±38.61	451.58^{a}±38.22	265.35^{b}±6.72	330.43^{a}±6.49	231.00^{b}±1.23	289.71^{b}±1.51
Medium 2	379.23^{b}±15.35	476.76^{a}±15.77	264.19^{b}±4.99	359.02^{a}±4.61	227.81^{b}±6.86	304.91^{a}±6.95
Medium 3	364.31^{b}±19.71	452.23^{a}±19.99	266.42^{b}±6.31	341.47^{a}±5.30	232.15^{b}±3.93	304.13^{a}±4.16
Medium 4	393.75^{b}±39.00	501.20^{a}±40.36	267.29^{b}±4.53	372.15^{a}±4.10	239.22^{b}±2.29	333.71^{a}±2.05

Rows bearing different superscripts within different types of oocyte differ significantly (P<0.05)

Table 2: Increase in diameter (µm) of oocytes with cumulus cells (mean±se) after *in vitro* maturation in different media.

Media	A	B	C
Medium 1	$83.02^{c}{}_{A}$±0.57	$65.08^{d}{}_{B}$±0.48	$58.70^{d}{}_{C}$±0.85
Medium 2	$97.53^{b}{}_{A}$±1.10	$94.83^{b}{}_{A}$±0.82	$77.10^{b}{}_{B}$±0.68
Medium 3	$87.91^{c}{}_{A}$±1.20	$75.05^{c}{}_{B}$±1.19	$71.98^{c}{}_{B}$±0.76
Medium 4	$107.45^{a}{}_{A}$±3.19	$104.86^{a}{}_{A}$±0.89	$94.48^{a}{}_{B}$±0.93

Column bearing different superscripts (a, b, c) differ significantly (P<0.05) Rows bearing different subscripts (A, B, C) differ significantly (P<0.05)

Discussion

In the present study, maturation performance of oocytes on the basis of expansion of cumulus cells was found to be 77.78, 81.44, 79.53 and 84.24 per cent in media 1, 2, 3 and 4 respectively. The variations were, however, non-significant. The present finding on IVM in TCM 199 with supplementation of kid serum in combination with oestradiol and FSH (medium 2) was comparable with the findings of (Tyagi *et al*. 1997) [15] who observed 80.60 per cent IVM of goat oocytes based on cumulus cells expansion in TCM-199 supplemented with FSH, oestradiol 17β and oestrous serum. The present data was also corroborated by the findings of (Yadav *et al*. 1998) [17] in goat, who cultured oocytes in TCM-199 supplemented with oestrous serum and reported 79.80 per cent maturation respectively based on cumulus expansion.

In the present study the mean diameter of the oocytes with cumulus cells or COCs differed significantly (P<0.05) between before and after IVM in all the media for 'A' and 'B' types of oocyte (Table 1) which indicated that the media used was efficacious in effecting cumulus cell expansion during IVM for good quality of oocytes (types A and B) surrounded by higher number of layer of cumulus cells. In case of 'C' type of oocytes, the mean diameter of the COCs although differed significantly (P<0.05) between before and after maturation in media 2, 3 and 4, it did not differ significantly in medium 1, *i.e*, control medium. This could indicate higher efficiency of the experimental media than the control medium since media 2, 3 and 4 could bring about significant changes in cumulus cell expansion in poorer quality oocyte (type 'C') while significant expansion of cumulus cells in 'C' type of oocyte was not achieved in the control medium. The increase in mean diameter of COCs in 'A', 'B' and 'C' types of oocyte was significantly (P<0.05) higher in medium 4 than in all other media (Table 2), which could indicate superiority of medium 4 that contained 17β oestradiol, FSH and LH in causing expansion of cumulus cells. Fukushima and Fukui (1985) [5] reported that the addition of FSH (2 IU/ml), LH (10µg/ml) and oestradiol (1µg/ml) to a medium improved the maturation and fertilizability of extra - follicular bovine oocytes cultured *in vitro*. Moor and Trounson (1997) [8] also demonstrated that FSH and LH administration was helpful in achieving ovine oocytes maturation *in vitro*. Saeki *et al.* (1990) [12] and Totey *et al.* (1992) [14] found that IVM performances of oocytes were higher in the presence of gonadotrophins compared to that without gonadotropins in the culture media. It was observed that LH acted *in vitro* on theca interna cells to stimulate androgen secretion and the secretion of androgen precursor by thecal cells which was the most important factor in determining the pre ovulatory increase in oestradiol production, Mariana *et al.* (2009)[7] was found to be stimulated by the increased frequency of episodic pulses of LH *in vivo* which might explain the efficacy of medium 4 that contained hLH in bringing about higher extent of cumulus cells expansion through better ability of IVM of COCs during incubation. Present finding could not be compared due to apparent lack of similar data in the literature reviewed depicting measurement of diameter of COCs before and after IVM.

The increase in mean diameter for all the types of oocyte was recorded to be significantly (P<0.05) higher in medium 2 than in medium 3 (Table 2), which suggested higher ability in cumulus expansion of medium 2 that contained FSH along with kid serum than medium 3 which was devoid of FSH although it contained kid serum. Addition of FSH was found to be beneficial in effecting oocyte maturation since *in vitro* stimulation by FSH improved the cAMP production by the COCs and induced the breakdown of the germinal vesicle (Guler *et al.* 2000) [6]. Except for 'A' type of oocytes between medium 3 and the control medium (medium 1), the increase in mean diameter of the different types of oocyte was significantly (P<0.05) lower in the control medium than in all other media (Table 2). Significantly (P<0.05) higher magnitude of expansion in 'A' type of oocytes that was surrounded by more layers of cumulus cells than in 'C' type of oocytes which was surrounded by lesser number of cumulus cells layer in all the media used (Table 2) indicated greater degree of maturation in 'A' grade oocytes. The present finding corroborated that of Bagger *et al.* (1993) [1] and Xia *et*

al. (1994) [16] who reported that cumulus might secrete an inducing substance that increased the maturation rate of oocytes. Cumulus morphology was linked to the maturational competence of oocytes Yuan *et al.* (2005) [18]. Cevik *et al.* (2011) [3] observed that oocytes showing no cumulus expansion following 24 Hours maturation was higher in poorer quality bovine oocytes in different treatment groups.

The increase in mean diameter of COCs did not differ significantly between 'A' and 'B' types of oocyte in media 2 and 4 (Table 2). This could suggest that media 2 and 4 could exert action for cumulus cell expansion in 'B' category of oocytes surrounded with lower number of cumulus cells layer comparable with that of 'A' type of oocytes having higher number of cumulus cells layer thus resulting in non-significant difference in expansion of cumulus cells between 'A' and 'B' types of oocyte. Except for medium 3, the increase in mean diameter of COCs in 'C' type of oocytes was significantly (P<0.05) lower than that of 'B' type of oocytes in all other media. This could be attributed to the status of the 'C' category oocytes which had the lowest number of surrounding cumulus cells layer. The non-significant difference in increase in mean diameter between 'B' and 'C' types of oocyte in medium 3 could be due to inefficacy of the medium in expanding cumulus cell layer of 'B' type of oocytes in contrast to media 2 and 4. The significant (P<0.05) difference in increase in mean diameters of 'A', 'B' and 'C' types of oocyte with cumulus cells in medium 1 (control medium) might be ascribed to its inability to cause cumulus cell expansion unlike the experimental media, *viz.*, medium 2, 3 and 4. It could be concluded that the ability of expansion of different types of oocyte and the efficacy of media in effecting the expansion of COCs could be revealed by objectively recording the diameters of COCs before and after IVM

Conclusion

The present study was conducted to study the goat oocytes maturation *in vitro* using different maturation media. The collected COCs were categorized as grade A, B and C depending on the compact cumulus cell layers surrounding the zona pellucida. The recovered oocytes were subjected to *in vitro* maturation (IVM) on incubation in four different maturation media *viz*. Medium-I (control), medium-II, medium-III, and medium-IV for 24 hours at 38.5^{o}C in 5 per cent CO_2 in humidified air. The maturation rate evaluated on the basis of cumulus expansion was 77.78, 81.44, 79.53 and 84.24 per cent in Medium-I, II, III and IV respectively the difference being statistically non-significant. The percentage of oocytes maturation based on cumulus cells expansion was found to be the highest (84.24) in medium-IV which consisted of TCM-199 supplemented with sodium pyruvate, L-glutamine, 10 per cent FBS, cysteamine (100µM/ml), 17β oestradiol (1µg/ml), pFSH (5µg/ml) and hLH (5µg/ml). The mean diameter of the oocytes with cumulus cells varied significantly (P<0.05) between before and after IVM in Media-I, II, III and IV for A and B types of oocyte. It also varied significantly (P<0.05) between before and after IVM in media-II, III and IV but not in medium I for C type of oocyte. It could be concluded that Slicing technique was found to be suitable for recovery of good quality goat oocytes and Medium consisting of TCM-199 supplemented with 17β oestradiol (1µg/ml), pFSH (5µg/ml) and hLH (5µg/ml) was found to be superior to other media used for IVM of goat oocytes.

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