Bangl. J. Vet. Med. (2024). 22 (2): 43 - 50 ISSN: 1729-7893 (Print), 2308-0922 (Online)

Received: 26-01-2025; Accepted: 04-03-2025 DOI: https//doi.org/10.33109/bjvmjd2024fam1

ORIGINAL ARTICLE

Determination of effective media and its hormone supplementation on *in vitro* maturation of crossbred cow oocytes

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Abstract

Background: The present experiment was conducted to determine an effective basic medium and its hormone supplementation for *in vitro* maturation of cow oocytes.

Methods: The experiment was conducted at the Department of Animal Nutrition, Genetics, and Breeding at Sher-e-Bangla Agricultural University, Dhaka- 1207, from January 2020 to December 2020. This study assessed the efficacy of Tissue Culture Media 199 (TCM-199) and TCM-199 supplemented with varying dosages of Gonadotropin (0.5, 1, 5, 10, and 15 µg/ml) hormone for the *in vitro* maturation of bovine oocytes. Synthetic ovurelin is used as a substitute of gonadotropin. Cumulus-oocyte complexes (COCs) were collected from cow ovaries by aspiration method and matured for 48 hours at 37.5ºC with 5% CO2 in humidified air in basic and supplemented media.

Results: Significantly higher ($p<0.05$) number of follicles were aspirated in ovaries without corpus luteum (CL) (4.59±0.27) compared with ovaries containing CL (3.05±0.13). Consequently, more COCs were found in ovaries without CL (4.61±0.25) than in ovaries with CL (1.98±0.16). The maturation rate was significantly higher ($p<0.05$) in (TCM-199 supplemented with gonadotropin 10 µg/ml) medium (65.71±2.74) than in other treatments. A higher maturation rate was found up to 10 µg/ml gonadotropin supplementation, but the maturation rate began to decline when the dose exceeded 10 µg/ml.

Conclusion: Therefore, ovaries without CL could be used in TCM-199, supplemented with Gonadotropin (10 µg/ml) as basic medium for optimal *in vitro* maturation rates of cow oocytes.

Keywords: Cow, ovaries, corpus luteum, maturation, gonadotropin.

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Introduction

In developing countries such as Bangladesh, cattle are an economically significant and promising animal. They have a significant influence in the achievement of economic growth. The share of livestock in the agricultural gross domestic product (GDP) at current prices was 16.33% in 2023-2024 (DLS,2024). Bangladesh, with a population of 160 million, consistently encounters challenges in meeting domestic milk and meat demand. The production capabilities of indigenous cattle breeds are either very low or not up to the mark. To address the increasing demand for meat and milk and to shorten the generation interval, the current cattle breed must be genetically upgraded through the application of Assisted Reproductive Technologies (ARTs), including *in vitro* fertilization (IVF), *in vitro* maturation

(IVM), multiple ovulation and embryo transfer (MOET), and *in vitro* embryo production (IVEP) (Rahman et. al., 2018). IVM of oocytes is the first and most important step to successful IVEP. Oocyte maturation refers to the processes that prepare the oocyte for fertilization and activate the mechanisms that govern pre-implantation embryonic development. The maturation of oocytes relies upon various factors such as the condition of ovaries, follicle size, oocyte quality, culture media, hormone supplementation, aspiration pressure during collection, and the interval between collection and processing of ovaries. Culture media plays a significant role in IVM of oocytes; ultimately proper fertilization and complete embryo development. The maturation rate varies greatly in different cultural media. The reasons for variation in maturation rate among studies might be due to variations in basic media and the amount of hormone or protein supplementation used for oocyte maturation (Rahman et. al., 2018). Across different species, multiple culture conditions have been used without a consensus of the optimal conditions for oocyte maturation (Bahrami *et al*., 2022). Different culture media such as TCM-199 (Kharche *et al*., 2006; Amer *et al*., 2008), minimum essential medium (MEM) (Ravindranatha *et al*., 2001), Ham's F-10 (Totey *et al*., 1993) have been used for IVM of mammalian oocytes elsewhere. Among them, TCM-199 is the most widely used culture medium for such purposes (Arunakumari *et al*., 2007). Supplementation of reproductive hormones in maturation media is essential because it improves the IVM rate of mammalian oocytes. Follicle-stimulating hormone, gonadotropin hormone and luteinizing hormone are commonly used hormones and significantly affect the IVM of cattle oocytes. In IVM, gonadotropins are the main stimulator of oocyte development, and FSH is deemed vital for the oocytes to become qualified to be *in vitro* fertilized (Lu *et al*., 2014; Khan *et al*., 2015). Even supplementation of the same hormone in different amounts has a conspicuous effect on the maturation rate and subsequent development after IVF. Therefore, the present study was undertaken to identify an effective basic medium and to determine the appropriate quantity of gonadotropin hormone supplementation in the basic medium for *in vitro* maturation of cow oocytes in Bangladesh.

Materials and methods

The experiment was conducted at the laboratory of the Animal Nutrition, Genetics, and Breeding Department of Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh.

Collection and processing of ovaries

The ovaries of native (local and cross-breed) cows with unknown reproductive status were collected from local slaughterhouses situated at Kaptanbazar, Krishimarket, Townhall, Dhaka. Ovaries were transported to the laboratory within a thermo-flask containing 0.9% physiological saline solution at 25–30°C within 2 to 3 hours of slaughtering the animals. In the laboratory, ovaries were rinsed thoroughly by physiological saline solution and recorded for the presence or absence of corpus luteum (CL). Then, each ovary was trimmed to remove the surrounding tissue and overlying bursa. A total number of 127 ovaries were collected; among them, 74 belonged to without CL and 53 with CL. 670 follicles were recorded on the surface of the ovaries. The number of aspirated follicles was 502.

Method of oocyte collection and grading

The number of visible follicles on the surface of different categories of ovaries was counted and recorded. The follicular fluid from 2 to 8 mm diameter follicles were aspirated using 18-gauge needle fitted with 10 ml syringe (Singha *et al*., 2015). The cumulus-oocyte-complexes (COCs) were searched and graded under microscope at 10x magnification. The COCs were classified into four grades on the basis of cumulus cells and nucleus (Khandoker *et al*., 2001). Those grades were Grade A: oocytes completely surrounded by

cumulus cells (Figure 1a); Grade B: oocytes partially surrounded by cumulus cells (Figure 1b); Grade C: oocytes not surrounded by cumulus cells (Figure 1c); and Grade D: degeneration observed both in oocytes and cumulus cells (Figure 1d). Grade A and Grade B considered as normal COCs; Grade C and Grade D considered as abnormal COCs.

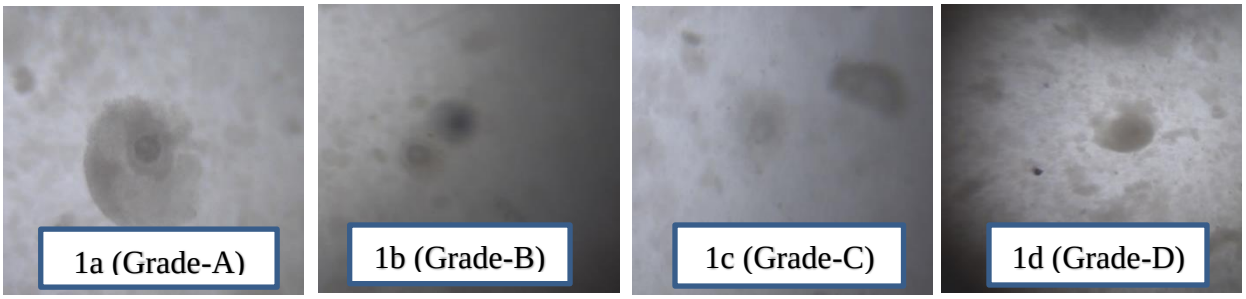

Figure 1. Grading of oocytes.

***In vitro* maturation (IVM) of COCs**

Only grade A and grade B COCs used for maturation. The maturation medium, TCM-199 (Sigma Chemical Co., USA), was prepared and divided into 5 groups. TCM-199 without any supplementation was used as a control, and TCM-199 was supplemented with varying doses of gonadotropin (0.5, 1, 5, 10, 15 µg/ml), respectively, to evaluate the effect of media and hormone. The pH of all media was adjusted to 7.4 by adding 1N NaOH on the day of oocyte collection and sterilized by passage through a 20 µm filter. About 2.5-3.5 ml of the medium from each group was poured into two 35 mm culture dishes. These culture dishes could be used to wash COCs. Then 4 drops of 100 µl of medium, depending on the number of oocytes, were poured into another culture dish; the COCs were transferred to the droplets and covered with paraffin oil (Labo America,

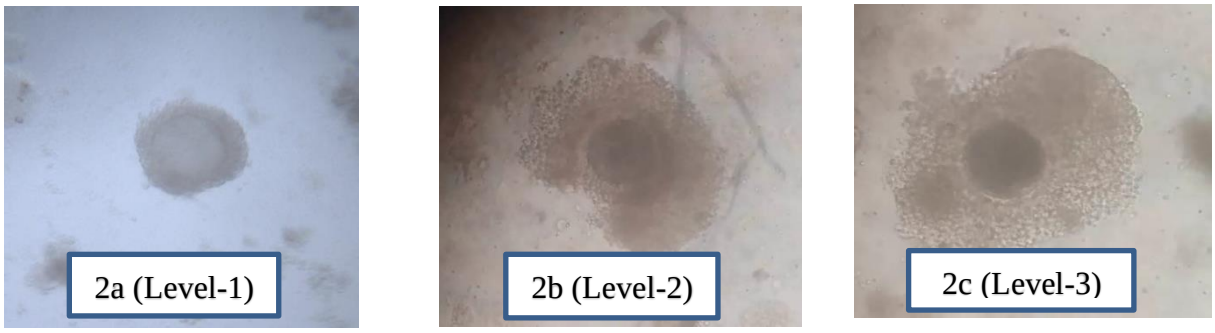

Figure 2. Different level of COCs expansion during *in vitro* maturation

Inc.,California, USA). Finally, the culture dish with droplets was kept in an incubator at 38.5 °C, with 5% CO2 in the humidified air for 48 hours. After 48 hours of culture of COCs in the maturation medium, cumulus expansion was determined on three levels under a microscope at 10x magnification (Talukder *et al*., 2011); level-1: cumulus cells less expansion (Figure 2a); level-2: cumulus cells moderate expansion (Figure 2b); level-3: marked expansion of cumulus cells with a compact layer or choronaradiata (Figure 2c).

Statistical Analysis

The difference between groups was assessed using Student's t-test, and all findings were presented as

Mean±SE. When the P-value was less than 0.05, the difference between the groups was regarded as significant. Using a statistical analysis system (SAS, 1999), Duncan's multiple range test (DMRT) was used to compare means.

Results and Discussion

Ovarian categories of collected samples

A total of 127 ovaries were collected from the slaughterhouse, and the oocytes were retrieved through the aspiration method. Among the 127 ovaries, 74 belonged to without CL group and 53 to with CL group. 670 follicles were recorded on the surface of the ovaries. The number of aspirated follicles was 502. Table 1 shows the difference between different parameters between the ovary with CL and the ovary without CL. Total number of visible follicles in ovaries without CL (6.00±0.28) was significantly higher ($p<0.05$) than that of CL-containing ovaries (4.34±0.13) and number of follicles aspirated in ovaries without CL (4.59±0.27) was significantly higher ($p<0.05$) than that of CL-containing ovaries (3.05±0.13). This result is similar to the previous study (Khandoker *et al*., 2017); which found a higher number of follicles were visible and aspirated from CL absent ovaries (11.2±1.8 and 37.8±14.9, respectively) compared to CL present ovaries 10.5±1.5 and 28.3±15.6, respectively). When the COCs were classified as normal and abnormal groups, significantly higher ($p<0.05$) number of normal COCs was found in ovaries without CL (3.08±0.21) than in ovaries with CL (0.69±0.09) and significantly higher ($p<0.05$) number of abnormal COCs was found in ovaries with CL (1.76±0.08) than ovary without CL (1.45±0.08) respectively which was similar to the previous study (Zaber *et al*., 2020); which found higher number of normal COCs in ovaries without CL (0.85±0.83) than ovaries with CL (0.75±0.85) respectively but non-significant. The total number of COCs in ovaries without CL (4.61±0.25) was significantly higher ($p<0.05$) than in ovaries with CL (1.98±0.16). This result is also similar to the previous study (Khandoker *et al*., 2012) which found significantly higher ($p<0.05$) number of total COCs in ovaries without CL (2.63±0.12) than ovaries with CL (1.81±0.17) respectively.

Table 1. Ovarian categories, number of follicles and collected COCs per ovary

Ovarian type	Total	Total number of visible follicles (Mean ±SE)	Number of follicles aspirated (Mean±SE)	Collected COCs per ovary (Mean± SE)		
				Normal Grade (A+B)	Abnormal Grade (C+D)	Total
Ovary with Corpus Luteum	53	4.34^{b}±0.13 (226)	3.05^{b}±0.13 (162)	0.69^{b}±0.09 (37)	1.76^{a}±0.08 (92)	1.98^{b}±0.16 (129)
Ovary without Corpus Luteum	74	6.00^{a}±0.28 (444)	4.59^{a}±0.27 (340)	3.08^{a}±0.21 (228)	1.45^{b}±0.08 (106)	4.61^{a}±0.25 (334)

Means with different superscripts within the column differ significantly (P<0.05). Figures in the parenthesis indicates the total number.

Determination of an effective basic medium for *in vitro* maturation (IVM) of oocytes

The result of *in vitro* maturation of COCs after 48 hours of cultured in different levels of gonadotropin hormone is presented in Table 2.

The maturation rates of oocytes were 42.25%, 52.50%, 60.13%, 62.25%, 65.71%, 61.15% in TCM 199 (control) and TCM-199 supplemented with different doses of Gonadotropins (0.5, 1, 5, 10 and 15 µg/ml). TCM-199 is one of the most widely used effective media for oocyte maturation. However, in this study, TCM-199 supplemented with the gonadotropin hormone was found to be more effective and had a noticeable influence on oocyte maturation. The maturation rate was significantly higher ($P<0.05$) in (TCM-199 + Gonadotropin 10 µg/ml) than TCM-199 (control) media. A gradual increase in maturation rate was found when the dose of Gonadotropin increased gradually up to 10µg/ml, but if the dose increased to 15 µg/ml, the maturation rate declined to (61.15±1.15%). In this experiment, 10 µg/ml Gonadotropin supplemented with TCM-199 was found to be the best medium for oocyte maturation (65.71±2.74%) at higher rate (Figure 3). This variation in maturation rate among studies might be due to variations in the amount of gonadotropin hormone supplementation with TCM-199 used for oocyte maturation. In IVM, gonadotropins are the main stimulator of oocyte development, and FSH is deemed vital for the oocytes to become qualified to be *in vitro* fertilized (Lu *et al*., 2014; Khan *et al*., 2015). The maturation rate is lower than previous study in indigenous zebu cows (Rahman *et al*., 2018; Singha *et al*., 2015). However, a comparatively lower maturation rate (53.8%) was reported in an earlier study on indigenous zebu cows (Morshed *et al*., 2014). The polar body extrusion rate of oocytes matured in TCM-199 was 55.6% (Sithole *et al*., 2023). The TCM 199 medium has been widely used for the maturation of oocytes in other species (Sithole *et al*., 2023) and the results obtained vary (Wang *et al*., 2007; Hoque *et al*., 2011) in goat. The maturation rate of oocytes was significantly higher ($P<0.05$) in FSH-supplemented TCM-199 than in gonadotropin supplementation (Rahman *et al*., 2018; Singha *et al*., 2015). The embryo development rate after IVF did not differ between bovine oocytes cultured in either FSH or gonadotropin-supplemented medium, despite the absence of a comparison of the IVM rate (Groza *et al*., 2008). Moreover, grades of oocytes may influence the *in vitro* maturation rates of oocytes as variation *in vitro* maturation rate was demonstrated between good and poor grade oocytes Goswami (2002). Supplementation of reproductive hormones in maturation media is essential because it improves IVM rate of mammalian oocytes (Rahman *et al*., 2018). The production and secretion of follicle Stimulating Hormone (FSH) and luteinizing Hormone (LH) are controlled by the Gonadotropin hormone. Both LH and FSH have a direct effect on the ovary; FSH stimulates follicle development, while LH induces ovulation and luteinization. FSH and (LH) are commonly added to maturation media to improve cumulus expansion known to be a predictor of oocyte maturation (Pandey *et al*., 2010). Gonadotropins are found to trigger cumulus cells to synthesize some molecules capable of initiating germinal vesicle breakdown (GVBD) as meiosis-activating sterols (Tsafriri *et al*., 2005).

Table 2. Effect of different maturation media on IVM rate of cow oocytes

Maturation medium	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%) (mean±SE)
TCM-199 (Control)	46	20	42.25^{f}±3.20
TCM-199+Gonadotropin (0.5 µg/ml)	40	21	52.50^{e}±2.11
TCM-199+Gonadotropin (1 µg/ml)	30	18	60.13^{d}±2.73
TCM-199+Gonadotropin (5 µg/ml)	25	14	62.25^{b}±2.60
TCM-199+Gonadotropin (10 µg/ml)	35	23	65.71^{a}±2.74
TCM-199+Gonadotropin (15 µg/ml)	40	25	61.15^{c}±1.15

Proportion values are mean ± SE. Maturation rates are significantly different from each other (P<0.05).

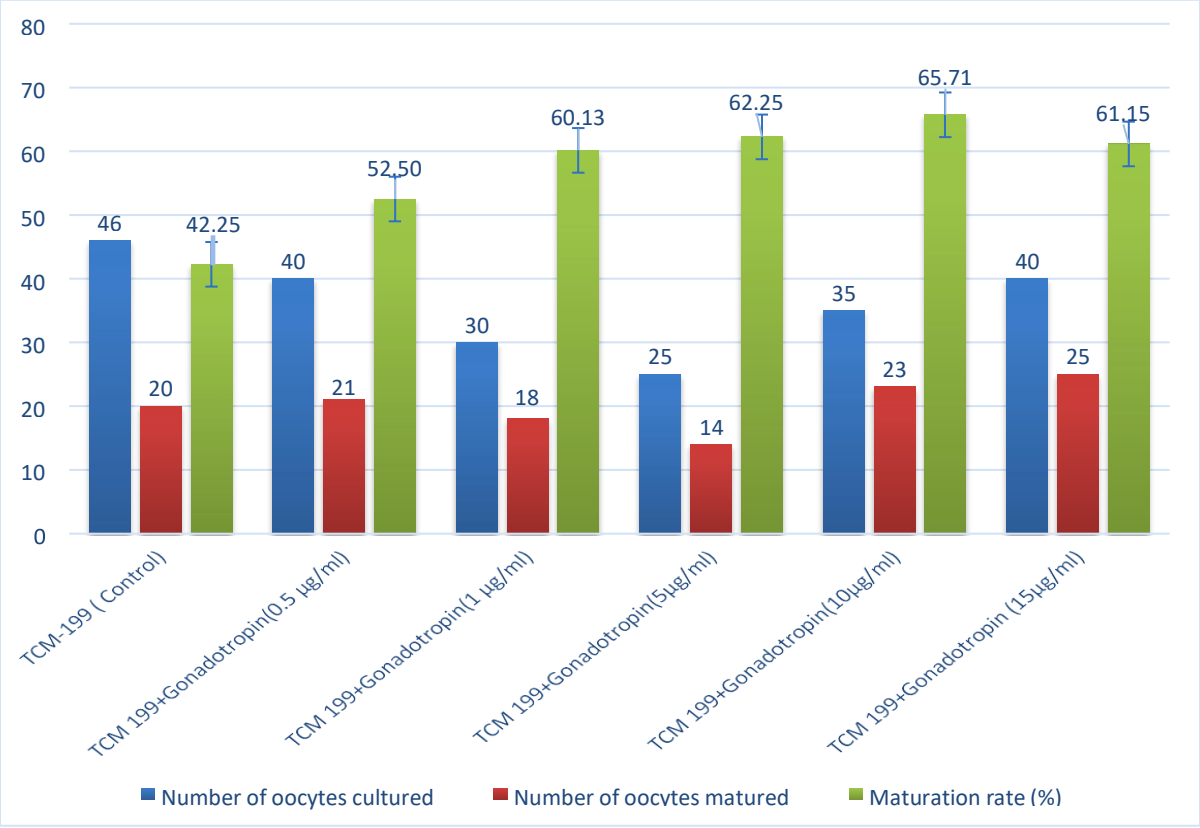

Figure 3. Effects of media on IVM rate of cow oocytes**.**

Conclusion

The result indicates that an ovary without CL is a good source of normal-grade oocytes for *in vitro* maturation of bovine oocytes. The maturation rate of oocytes in media TCM-199 supplemented with Gonadotropin hormone 10 µg/ml showed the best result compared to control and other treatment doses. It can be concluded that normal-grade oocytes from ovaries without CL could be used in TCM-199 basic medium supplemented with Gonadotropin (10 µg/ml) for optimal *in vitro* maturation rate of bovine oocytes.

Acknowledgment

The Ministry of Science and Technology, People's Republic of Bangladesh, is acknowledged for providing a research fellowship.

Conflict of Interest

The authors declare no conflict of interest.

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