Ann. Bangladesh Agric. (2022) 26 (1) : 1-13 *ISSN 1025-482X (Print)*
www.doi.org/10.3329/aba.v26i1.67013 07 *2521-5477 (Online)*

REPRODUCTIVE PHASES INFLUENCE ON *IN VITRO* MATURATION OF ABATTOIR-DERIVED BOVINE OOCYTES

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Abstract

This study investigated the influence of the reproductive phase of oocyte donors originating from abattoir-derived ovaries on *in vitro* maturation (IVM) of bovine oocytes. The IVM of the oocytes was evaluated by observing the degree of cumulus cell (CC) expansion and extrusion of the first polar body (PB-1) as well as both nuclear progression (NP) and cytoplasmic maturation (CM). For NP, after 24 h of IVM, oocytes were denuded and stained with DNA fluorochrome and observed under an epifluorescence microscope. The male or female pronucleus (PN) forming ability of an oocyte following *in vitro* fertilization (IVF) was considered as CM. For CM, the fertilized zygotes after 24 h post-insemination were stained with DNA fluorochrome and observed under an epifluorescence microscope. Results revealed that the maturation rate was significantly influenced by the reproductive phase of the oocytes donor, luteal phase ovaries (LPO) versus follicular phase ovaries (FPO) based on the presence or absence of corpus luteum (CL), respectively. The significantly higher CC expansion (2°; 44.9±4.9% versus 35.9±2.7%) and PB-1 extrusion (58.3±1.7% versus 46.7±2.4%) were observed with LPO than FPO oocytes, respectively. A significant ($p<0.05$) effect of the reproductive phase was also noted with LPO oocytes progressing higher rate of mature metaphase II stage (MII; 58.3±1.7% versus 46.7±2.4%) and PN (54.9±2.3% versus 44.4±3.0%) formation following IVF than FPO oocytes that imply better NP and CM of oocytes, respectively. Therefore, the present study indicates that the reproductive phase of oocyte donors notably from LPO positively influences the IVM of bovine oocytes to produce a higher number of viable embryos *in vitro.*

Keywords: Bovine, abattoir, reproductive phase, oocyte, *in vitro* maturation.

Introduction

Abattoir-derived ovaries constitute a cost-effective source of oocytes for *in vitro* production (IVP) of embryos for commercial and research purposes. However, such oocytes are extremely heterogenous in terms of quality and developmental competence (Lonergan *et al.,* 1994; Gupta *et al.,* 2007). The reduction of variability of the oocyte quality is very important when they are selected as recipients for somatic cell nuclear transfer (SCNT) to produce genetically modified animals or for research purposes (Das *et al.,* 2010). Variable results have been described for *in vitro*

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maturation (IVM), *in vitro* fertilization (IVF) and subsequent embryonic development for the oocytes collected from ovaries at different stages of the reproductive cycle in cattle (Racedo *et. al.,* 2008), pig (Bagg *et al.,* 2006; Gupta *et al.,* 2007), human (Sánchez *et al.,* 2017), cat (Karja *et al.,* 2002) and dog (Evecen *et al.,* 2010). In light of these previous reports, therefore, suggest the importance of the source of oocytes in determining the IVM and subsequent developmental competence of embryos.

The *in vitro* embryo production (IVEP) is an established technology in developed countries and is routinely used to produce genetically superior animals for meat and milk production and also conservation of rare and endangered wild animals (Das *et al.,* 2010). The flurry of research activities on IVEP have been done last few decades throughout the world (Parrish *et al.,* 1988; Brackett *et al.,* 1993; Krisher *et al.,* 1999; Gandhi *et al.,* 2000; Galli *et al.,* 2001; Racedo *et. al.,* 2008). The IVEP is a multistep methodology comprising the following procedures: i) IVM of oocytes, ii) IVF or co-incubation of capacitated spermatozoa with IVM oocytes and iii) *in vitro* culture (IVC) of zygotes up to the blastocyst stage.

The IVM of the oocyte for embryo production is the first and most determinant step of embryo viability and is used to improve the production of genome-modified domestic animals (Jin *et al.,* 2018). The maturation of mammalian oocytes is defined as the sequence of events occurring from the germinal vesicle stage for the completion of the second meiotic division with the formation of the first polar body or PB-1 (Paramio and Izquierdo, 2014). The importance of oocyte quality, one of the most important intrinsic factors involved in the developmental competence of embryos, might be more appropriate to determine the oocyte's nuclear and cytoplasmic maturation, which are attained during its growth in the follicle (Gandolfi and Gandolfi, 2001; Gupta *et al.,* 2007). A competent oocyte is by definition able to sustain embryonic development to term (Gibbons *et al.,* 2007). Nuclear maturation is characterized by the oocyte's ability to resume meiotic division up to metaphase II during IVM. Nuclear maturation (NP) can be visualized by the extrusion of PB-1 and the appearance of the metaphase plate using a nuclear staining technique such as Hoechst 33342 (Das *et al.*, 2014) or orcein (Liu *et al.*, 2003). Cytoplasmic maturation (CM) is dictated by the entire array of maternal mRNAs, proteins, substrates, nutrients and mitochondrial accumulation in the ooplasm during folliculogenesis (Gandolfi and Gandolfi, 2001). Importantly, CM enables the oocytes to control the first cleavage divisions until the embryonic genome is activated and takes over this responsibility (Das *et al.*, *Unpublished data*). Follicular diameter (Lonergan *et al.*, 1994; Sanchez *et al.,* 2017), follicle status (Gupta *et al.*, 2007), oocyte diameter (Racedo *et al.*, 2008), cumulus morphology (Rahman, 2008) and reproductive status of animals (Gupta *et al.*, 2007) are some factors that have been linked to the maturational competence of oocytes. To the best of our knowledge, only a few research groups (including our research group) are currently conducting research in this area of assisted reproductive techniques (ARTs) in Bangladesh. All the researchers are still struggling to optimize and establish standard culture conditions for IVM, IVF and IVC of cow oocytes in Bangladesh with

limited success (Rahman, 2003; Das, 2005; Islam *et al.*, 2007; Talukder *et al.*, 2008; Dev *et al.,* 2012; Morshed *et al.,* 2014) and goat (Rahman, 2008; Rahman *et al.* 2009; Khatun *et al.,* 2011; Hoque *et al.,* 2011). Therefore, the present study investigated the effect of the reproductive phase of oocyte donors originated from abattoir-derived ovaries on IVM of bovine oocytes.

Materials and Methods

Chemicals and reagents

Unless otherwise indicated, all the chemicals and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Study location

The study was conducted at the Reproductive Biotechnology Laboratory, Department of Gynecology, Obstetrics & Reproductive Health (GOR), Faculty of Veterinary Medicine & Animal Science (FVMAS), Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU) during the period from January to December 2018.

Collection and transportation of ovaries

Bovine ovaries were collected in 0.9% saline solution at 35–37ºC from local abattoirs of Gazipur City Corporation (GCC), Gazipur and Dhaka South City Corporation (DSCC). The collected ovaries were then transported to the laboratory within 2–3 hours (h) of collection. The overall experimental approaches are presented in Fig.1.

Collection and selection of cumulus-oocyte complexes

The ovaries were rinsed 4 times in saline solution at 35–37°C. The abattoir-derived heterogenous population of bovine ovaries was grouped into two categories, namely luteal phase ovaries (LPO) and follicular phase ovaries (FPO) based on the presence or absence of corpus luteum (CL), respectively. Follicles of 2–8 mm diameter were aspirated separately from both LPO and FPO groups of ovaries with an 18-gauge hypodermic needle (Terumo Corporation, Tokyo, Japan) attached to a 10-mL disposable syringe (JMI Disposable Syringe, JMI Syringe & Medical Devises Ltd., Cumilla, Bangladesh). The follicular fluid was left for 5 minutes (min) and the sediment is transferred in a 60 mm Petri dish. The retrieved follicular aspirates were then diluted with TL-HEPES and searched for cumulus-oocyte complexes (COCs) under a stereomicroscope. The collected COCs from both LPO and FPO groups were washed three times in fresh TL-HEPES and once in the maturation medium before putting those in the maturation drops. Oocytes with multi-layered compact cumulus investment and homogenous ooplasm were selected for *in vitro* maturation (IVM).

In vitro maturation of COCs

The selected good quality COCs were matured in the tissue culture media 199 (TCM 199) supplemented with sodium pyruvate (0.25 mM), fetal bovine serum (FBS, 10%, v/v), bovine FSH (0.05 µg/mL), LH (5 µg/mL), estradiol (1 µg/mL) and gentamycin (50 µg/mL) in 50 µl drops on 35 mm culture Petri dish overlaid with mineral oil at 39ºC in an incubator with 5% CO_2 in humidified air for 22–24 h. IVM of these COCs was evaluated by observing cumulus cell (CC) expansion and first polar body (PB-1) extrusion as described earlier by Gupta *et al*. (2007). The IVM ability of COCs was also evaluated by

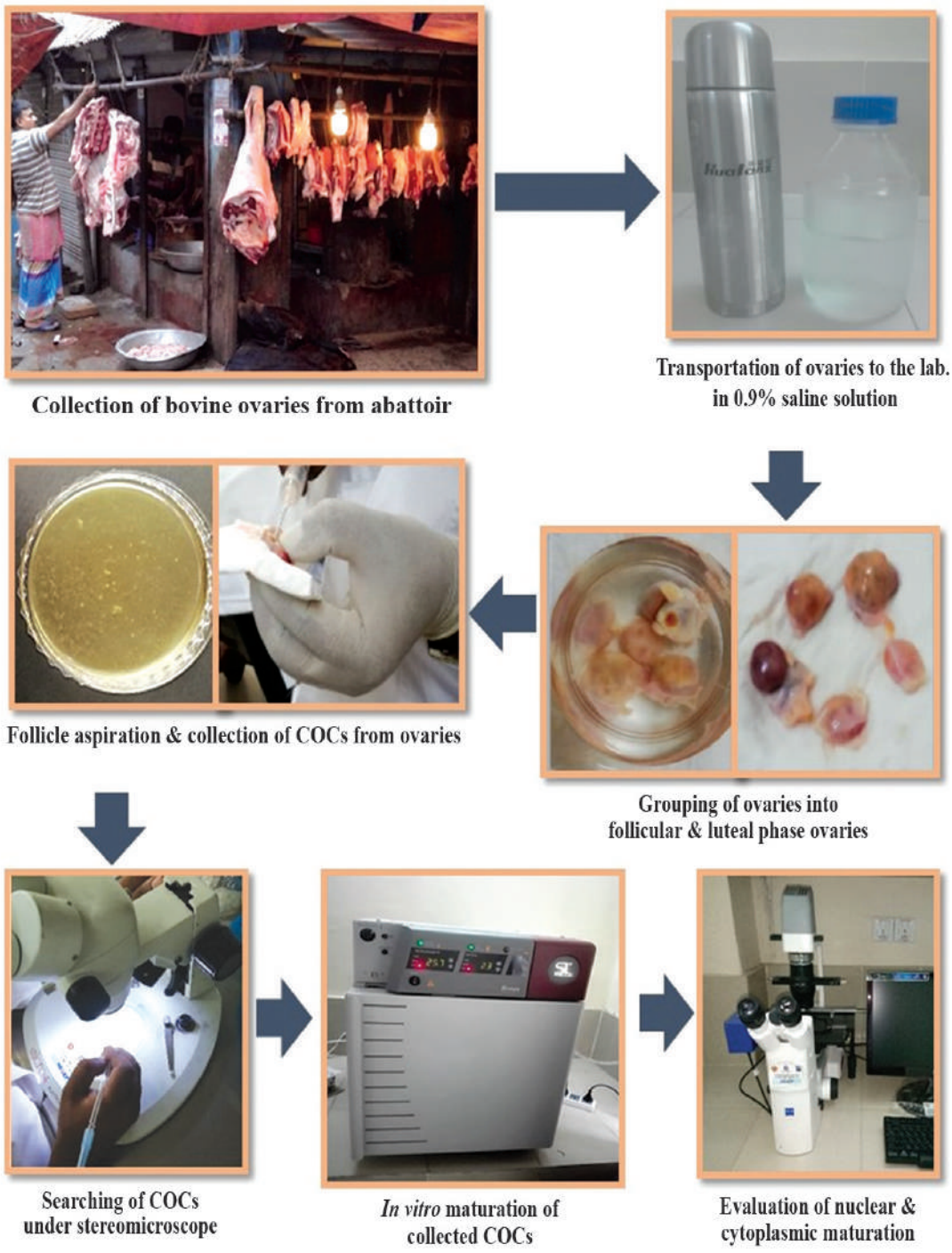

Fig. 1. Photographic representation of experimental approaches for the collection of an abattoir-derived bovine ovaries, cumulus-oocyte complexes (COCs) and *in vitro* maturation.

nuclear progression (NP) after 24 h of IVM culture and cytoplasmic maturation (CM) following 6 h of *in vitro* fertilization (IVF) by observing pronucleus (PN) formation.

Fluorescent staining for assessment of nuclear progression following IVM

Nuclear progression (NP) of bovine IVM oocytes was performed using Hoechst 33342 staining as described by Das *et al.* (2014). Briefly, IVM bovine oocytes were stripped of CC in TL-HEPES supplemented with 0.1% hyaluronidase and washed 3 times using fresh TL-HEPES contained with 3 mg/mL of BSA. Denuded oocytes were fixed for 5 min in a fixative solution that contained 2% formalin and 0.25% glutaraldehyde, mounted on clean glass slides and stained with a glycerol-based Hoechst 33342 (12.5 µg/mL) staining solution for 10 min. The stained nuclei that appeared blue when visualized under UV light illumination were then assessed for nuclear status (Fig. 3) in each oocyte and digital images (Nikon, Nikon Corporation, Tokyo, Japan) were taken.

Semen and sperm preparation

Frozen semen from Holstein-Friesian sires was used in this study. Semen straws were purchased from American Dairy Limited, Sreepur, Gazipur. Briefly, two straws (0.25-mL straws, 25×10^6 live sperm/straw) from each bull were thawed at 37°C for 12–15 seconds (sec). The thawed semen was washed three times in TL-HEPES by centrifugation at 800 rpm for 10 min. The washed sperm were subjected to swim-up in a 5 mL cap tube for 20 min in the CO_2 incubator in an inclined position to collect the most motile and purified sperm.

***In vitro* fertilization**

A modified Tyrode's lactate solution, Fert-TALP supplemented with fatty acid-free BSA (6 mg/mL) and sodium pyruvate (0.25 mM) was used for sperm-oocyte co-incubation. Ten to fifteen matured oocytes were transferred to individual drops (44 µL) of Fert-TALP. Two-µL heparin (5 µg/mL) and 2 µL PHE (D-penicillamine, 20 µM; hypotaurine, 10 µM; and epinephrine, 1 µM) added to each of the IVF drops. Two µL of sperm suspension were added to an IVF drop; thus, the final drop became 50 µL. The final concentration of spermatozoa in the drop was 2×10^6/mL spermatozoa and COCs were co-incubated for 6 h at 39°C with 5% CO_2 in humidified air. After 6 h of sperm-oocyte co-incubation, the presumptive zygotes (fertilized oocytes) were cultured. The culture medium was M-199 (with sodium bicarbonate, 26.2 mM as a buffer against 5% CO_2 in air) supplemented with sodium pyruvate (0.25 mM), fatty acid-free BSA (6 mg/mL) and Gentamycin (25 µg/mL). The sperm-oocyte co-incubation culture was performed in 50 µL drops prepared with the culture media in 35 mm Petri dish.

Fluorescent staining for assessment of cytoplasmic maturation

Cytoplasmic maturation (CM) of bovine IVM oocytes was performed using Hoechst 33342 staining as described by Das *et al.* (2010). Briefly, IVM bovine oocytes and spermatozoa were co-incubated for 6 h at 39°C with 5% CO_2 in humidified air. After sperm-oocyte co-incubation, the presumptive zygotes were cultured for an additional 10–12 h. At 16-

18 h post-insemination, the presumptive zygotes were fixed for 5 min in a fixative solution that contained 2% formalin and 0.25% glutaraldehyde, mounted on clean glass slides and stained with a glycerol-based Hoechst 33342 (12.5 µg/mL) staining solution for 10 min. The ability of male and female PN formation in a fertilized oocyte is the determinant of oocyte CM (Das *et al.,* 2010; Jia and Wang, 2020). The stained male and female PN, which appeared blue when visualized under UV light illumination was then assessed for CM (Table 3) in each oocyte and digital images (Nikon, Nikon Corporation, Tokyo, Japan) were taken.

Statistical analysis

The data generated from this experiment were entered into Microsoft Excel (2016) worksheet, organized and processed for further analysis. Analysis was performed with the help of Statistical Packages for Social Sciences, version 20.0 for windows (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used to describe the basic features of the data in the present study. Chi (χ^2) squared test and one-way ANOVA were used to assess the significant ($p<0.05$) difference between the groups.

Results and Discussion

Reproductive phase influences the cumulus cell expansion and first polar body extrusion during *in vitro* maturation of bovine oocytes

In vitro maturation (IVM) rate based on the cumulus cell (CC) expansion and first polar body (PB-1) extrusion of bovine cumulus-oocyte complexes (COCs) or oocytes originated from both the luteal phase ovaries (LPO) and follicular phase ovaries (FPO) groups is summarized in Table 1. The IVM of bovine oocytes was assessed by observing the degree of CC expansion (Fig. 2) and the extrusion of PB-1 (Table 1 and Fig. 3). The degree of CC expansion was categorized into three groups namely 0, 1 and 2 degrees. The percentages of CC expansion like 0, 1 and 2 degrees in FPO were 30.3±1.1, 33.8±3.1 & 35.9±2.7%, respectively and LPO was 23.7±2.2, 31.4±4.4 & 44.9±4.9%, respectively. The 2° CC expansion rate was significantly higher in LPO-derived oocytes than in FPO-derived oocytes. On the contrary, 0° CC expansion was lower significantly in LPO-derived oocytes than in FPO-derived oocytes. However, no difference was observed in 1° CC expansion in the studied groups. The higher percentages of PB-1 extrusion (58.3±1.7%)

Table 1. Effect of oocyte source on the IVM rate (mean ± SEM) of bovine oocytes based on the degree of CC expansion and PB-1 extrusion

Oocyte source	No. of oocytes	Degree of CC expansion			Extrusion of PB-1
		0	1	2	
FPO	119	30.3±1.1^a (36)	33.8±3.1^a (41)	35.9±2.7^a (42)	46.7±2.4^a (55)
LPO	92	23.7±2.2^b (23)	31.4±4.4^a (29)	44.9±4.9^b (40)	58.3±1.7^b (53)

Four replicates were used. FPO = Follicular phase ovaries, LPO = Luteal phase ovaries. Values with the same letters within a column denote a non-significant difference ($p<0.05$). Values within parentheses indicate the number of oocytes.

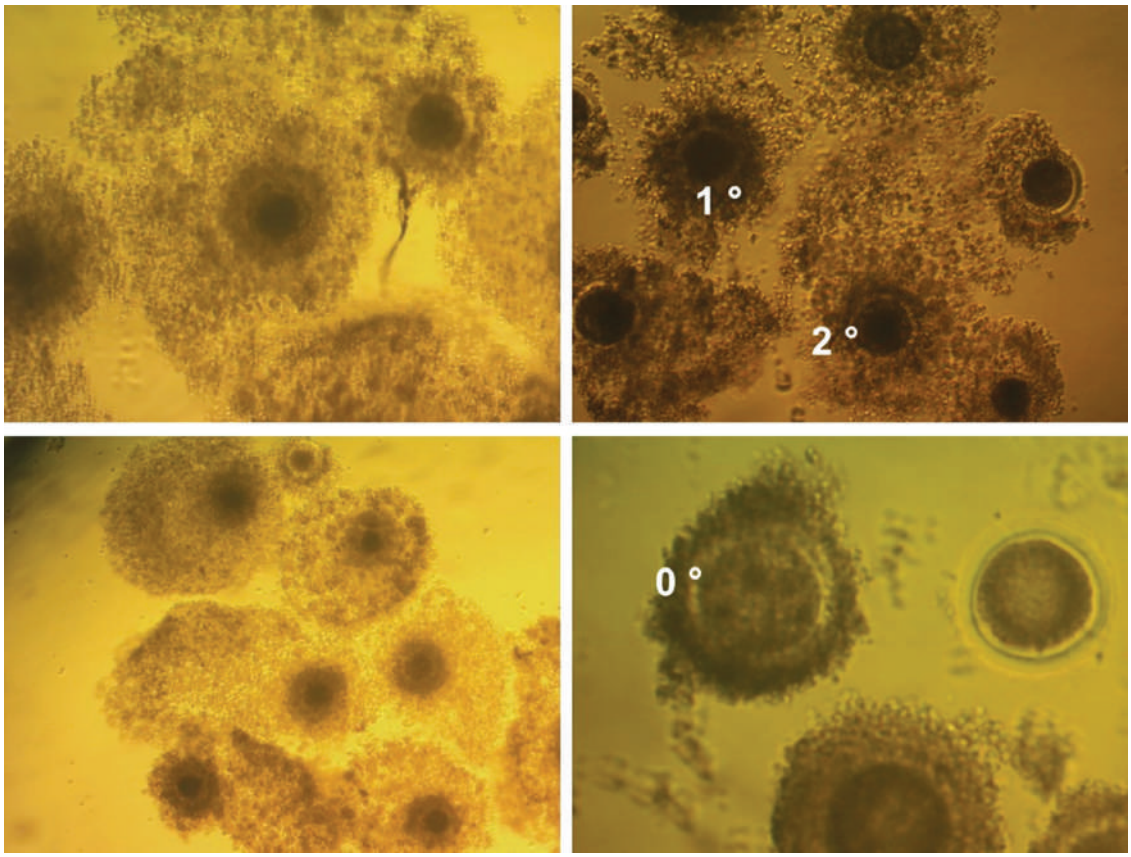

Fig. 2. Photographic representation of IVM of abattoir-derived bovine COCs/oocytes observed under a stereomicroscope. Different degrees of CC expansion of COCs (0-degree, 1 degree and 2 degrees); degree 2 (full CC expansion): all CC homogeneously expanded; degree 1 (moderate CC expansion): 70% CC homogeneously expanded; degree 0 (slight or no expansion): CC were highly adherent to the zona pellucida. Oocytes with CC expansion of degrees 1 and 2 were considered mature.

were observed in LPO-derived oocytes than in FPO-derived oocytes (46.7±2.1%), which differ significantly. These results indicated that the reproductive phase influences the IVM ability of bovine oocytes. Oocyte quality is associated with follicle size (Sanchez *et al.,* 2017; Jia and Wang, 2020). Bovine oocytes from small antral follicles (<3 mm diameter) have low competence to be matured *in vitro* and to assure early embryo development. Therefore, bovine oocyte from medium antral follicles (3–8 mm) are commonly used for IVM and *in vitro* embryo production or IVEP (Lequarre *et al.*, 2005; Jia and Wang, 2020). In the current study, oocytes obtained from LPO-derived ovaries originated mostly from medium and large-sized follicles; whereas, FPO-derived oocytes originated mainly from small-sized follicles due to a smaller number of medium-sized follicles present in FPO-derived ovaries resulted in significantly lower CC expansion and PB-1 extrusion ability than LPO-derived oocytes.

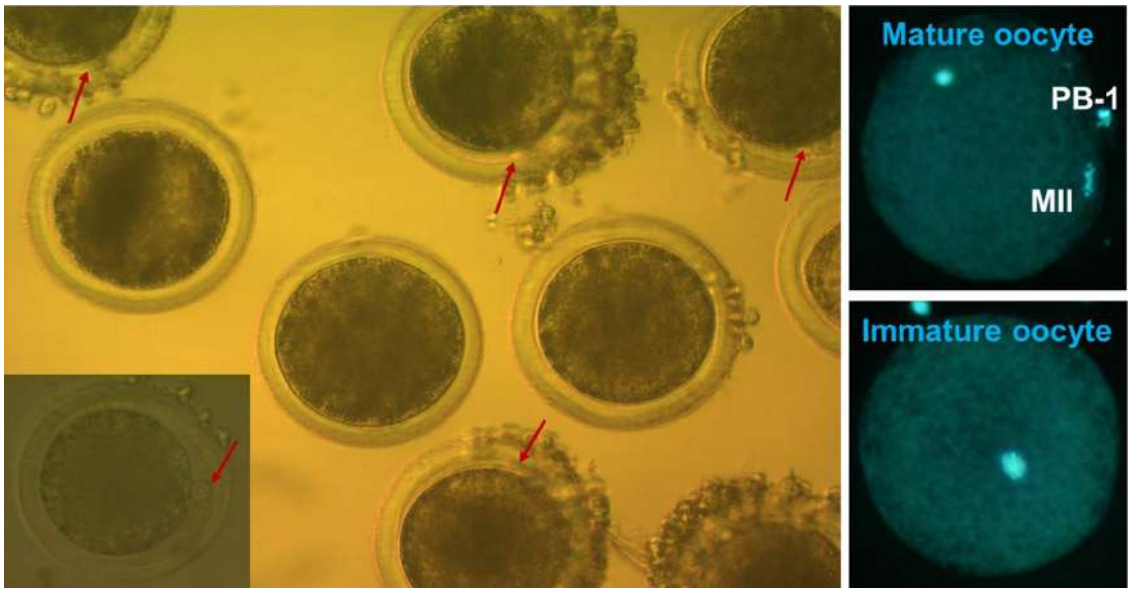

Fig. 3. Assessment of IVM of abattoir-derived bovine oocytes after 22–24 h of culture. Arrow indicates the extruded PB-1. Nuclear maturation of bovine oocytes was assessed by Hoechst 33342 staining and observed with Nikon Inverted microscope with fluorescent facilities (Using a DAPI filter).

The results obtained in the current study are consistent with the previous reports (Lequarre *et al*., 2005; Jia and Wang, 2020).

Several previous reports also suggested the importance of the source of oocytes in determining the developmental competence of embryos and variation in the experimental results due to ovary source (Racedo *et al.,* 2008; Evecen *et al.,* 2010). Variable results have also been described for IVM, IVF and subsequent embryonic development for the oocytes collected from ovaries at different stages of the reproductive cycle in several species including bovine (Sanchez *et al.,* 2017). The results obtained in the present study are consistent with several previous findings (Gupta *et al.,* 2007; Racedo *et al.,* 2008). However, in their studies, Bagg *et al.* (2006) found no differences between oocytes from CL and non-CL-bearing ovaries, obtained from peri-pubertal pigs, for rates of meiotic maturation, cleavage and blastocyst formation following parthenogenic activation. This difference might be species specific and/or experimental conditions and culture media used in that study.

Reproductive phase impacts the nuclear maturation of IVM bovine oocytes

This study evaluated the effect of the reproductive phases on nuclear progression (NP) following IVM of bovine oocytes. The oocytes nucleus at different stages of NP namely, Germinal Vesicle (GV), Germinal Vesicle Breakdown (GVBD), Metaphase I (MI), Anaphase-I-Telophase-I (AI-TI) and Metaphase II (MII) stages stained blue when stained with DNA fluorochrome, Hoechst 33342 and visualized under UV light illumination. The result of different stages

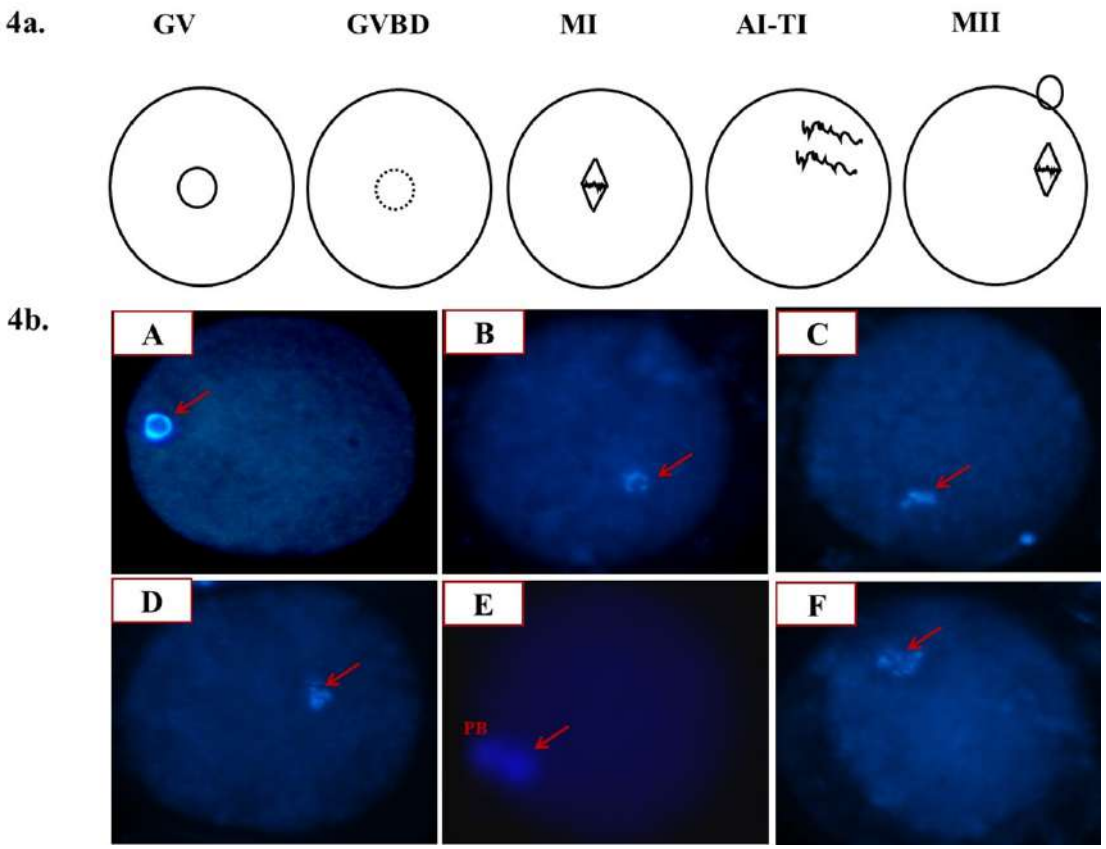

Fig. 4. **Nuclear progression of bovine *in vitro* matured oocytes retrieved from abattoir-derived bovine ovaries. 4a) Graphical representation of NP during oocyte meiotic maturation. 4b) NP of oocytes meiotic maturation stained with DNA fluorochrome, Hoechst 33342. A. GV stage; B. GVBD stage; C. MI stage; D. AI-TI stage; E. MII stage, PB = Polar body and F. Degenerated. Arrow indicates the status of nuclear progression. GV = Germinal vesicle; GVBD = Germinal vesicle breakdown; MI = Metaphase I; AI-TI = Anaphase I-Telophase I and MII = Metaphase II.**

of NP following IVM of bovine oocytes is summarized in Table 2.

The NP of oocytes derived from FPO and LPO were 11.6±2.2% GV; 11.8±0.7% GVBD; 14.8±3.1% MI; 10.1±2.3% AI-TI; 46.7±2.4 % MII stage and 9.8±0.2% GV; 11.1±1.3% GVBD; 8.6±1.2% MI; 8.5±1.7% AI-TI; 58.3±1.7% MII stage, respectively following 22-24 h IVM. The MII stage oocytes were significantly higher in the LPO group than that of the FPO group indicating better maturation ability of oocytes retrieved from LPO. The results obtained in the current study are partially consistent with the previous results, where authors described that IVM of oocytes was not influenced by the reproductive phase of oocytes donor, but influenced the developmental ability to the blastocyst stage of the subsequent zygotes originated from LPO-derived oocytes (Gupta *et al.,* 2007; Racedo *et al.,* 2008). It might be implied that, under

Table 2. Nuclear progression (mean±SEM) following IVM of bovine oocytes derived from follicular and luteal phase ovaries

Groups	No. of oocytes	GV	GVBD	MI	AI-TI	MII	Degenerated
FPO	119	11.6±2.2[a] (14)	11.8±0.7[a] (14)	14.8±3.1[a] (18)	10.1±2.3[a] (12)	46.7±2.4[a] (55)	5.0±0.8[a] (6)
LPO	92	9.8±0.2[a] (9)	11.1±1.3[a] (10)	8.6±1.2[a] (8)	8.5±1.7[a] (8)	58.3±1.7[b] (53)	3.6±1.4[a] (4)

Four replicates were used. Values in the superscript within the same column indicate significant differences ($p<0.05$). Values in the parenthesis indicate the number of oocytes in each stage. FPO = Follicular phase ovaries, LPO = Luteal phase ovaries.

the culture system used in those studies, the hormonal environment of the donor female at the time of ovary retrieval do not influence its potential for meiotic maturation.

Reproductive phase effects the cytoplasmic maturation of IVM bovine oocytes

The pronucleus (PN) formation either male or female PN ability of an oocyte following IVF was considered as CM (Blondin *et al*., 1997). The data of CM of bovine oocytes are summarized in Table 3. The results showed that the CM of bovine oocytes originating from FPO and LPO were 44.4±3.0 and 54.9±2.3%, respectively which differed significantly. However, bovine oocytes originating from LPO showed higher one male and one female PN (1 MPN+1 FPN; 31.4±1.6%) formation ability than that of FPO (21.5±3.0%). On the contrary, bovine oocytes originating from LPO showed lower more than one male and one female PN (>1 MPN+1 FPN; 19.7±0.9%, FPN 3.8±1.4%) and FPN formation ability than that of FPO (18.4±2.7%, 4.5±1.8%).

Blondin *et al.* (1997) reported that the decrease in the developmental competence of *in vitro*-matured bovine oocytes may be partly due to asynchronous nuclear and cytoplasmic maturation, leading to insufficient cytoplasmic maturity. Likewise, another study by Jia and Wang, (2020) showed that the novel IVM system based on C-type natriuretic peptide (CNP)-pretreatment significantly improved

Table 3. Cytoplasmic maturation of *in vitro* matured bovine oocytes retrieved from abattoir-derived bovine ovaries with respect to the estrous cycle phases

Reproductive phases	No. of Oocytes	No PN formation	Cytoplasmic maturation			
			MPN+FPN	>1 MPN+FPN	FPN	Total
FPO	90	55.6±3.0[a] (50)	21.5±3.0[a] (20)	18.4±2.7[a] (16)	4.5±1.8[a] (4)	44.4±3.0[a] (40)
LPO	87	45.1±2.5[b] (40)	31.4±1.6[b] (27)	19.7±0.9[a] (17)	3.8±1.4[a] (3)	54.9±2.3[b] (47)

Four replicates were used. Values in the superscript within the same column indicate a significant difference ($p<0.05$). Values in the parenthesis indicate the number of oocytes. FPO = Follicular phase ovaries, LPO = Luteal phase ovaries, MPN = Male pronucleus and FPN = Female pronucleus.

the developmental competence of bovine oocytes from medium-sized follicles with a diameter of 3–8 mm. The mechanisms by which CNP improved bovine oocyte CM might be due to elevated levels of maternal mRNA and proteins during meiotic arrest *in vitro*, resulting in an increased rate of embryo development beyond the "maternal-to-embryonic transition" stage. The results of the present study showed that FPOs had a higher proportion of smaller-sized follicles, whereas LPOs possessed a higher proportion of medium or large-sized follicles. Given that bovine oocytes originating from small-sized follicles have lower CM ability *in vitro* than oocytes originating from medium or large-sized follicles. Similarly, it is also likely that the presence of a larger number of oocytes from smaller follicles might have resulted in overall low CM in FPO-derived oocytes. The lower CM of FPO-derived oocytes in the present study might also be ascribed to their reduced ability to accumulate cAMP during IVM (Bagg *et al*., 2006; Jia and Wang, 2020). Our results are consistent with previous studies, where authors reported that ovaries from pregnant cows with CL had high-quality oocytes with great developmental competence (Moreno *et al*., 1993; Jia and Wang, 2020).

Conclusion

The maturation ability of abattoir-derived bovine oocytes was significantly higher in regard to cumulus cell expansion and first polar body extrusion in the luteal phase ovaries (LPO) than in the follicular phase ovaries (FPO) group. The maturation rate was also higher in LPO than FPO group in regard to nuclear progression to the mature metaphase II stage oocytes after *in vitro* maturation (IVM) and higher cytoplasmic maturation as revealed by pronucleus formation ability following *in vitro* fertilization. Therefore, the present study indicates that the reproductive phase of oocyte donors notably from LPO positively influences the IVM of bovine oocytes to produce a higher number of viable embryos *in vitro.* However, further studies are needed for the optimization of embryo culture processes before going to *in vitro* production of embryos and embryo transfer to recipient animals for the production of genetically superior live offspring.

Acknowledgments

The authors sincerely acknowledge the Research Management Wing (RMW) of Bangabandhu Sheikh Mujibur Rahman Agricultural University for the funding of the project entitled 'Production of Live Calf with *in vitro* Produced Embryos from Abattoir-derived Bovine Oocytes in Bangladesh' for the period of 2017-2020.

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