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Comparison of the Efficacy of Serum Gonadotrophin, Estrodiol-17β, Estrous Buffalo Serum and Buffalo Follicular Fluid Supplementation on *invitro* Cytoplasmic Maturation Rate of Buffalo Oocytes

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A B S T R A C T

An experiment was conducted to compare the efficacy of serum gonadotropin @50 and 100IU/ml, estrodiol-17β @0.5 and 1µg/ml, EBS @5 and 10% and BFF @5 and 10% supplementation on *in vitro* cytoplasmic maturation rate of buffalo oocytes. Ovaries were collected from buffaloes slaughtered at local abattoir. Oocytes were aspirated and good and excellent quality oocytes were matured in basic maturation media TCM199 supplementing above mentioned supplements separately. The recovery rate of culturable oocytes was 85.33%. The cytoplasmic maturation rate (CMR) was highest in the oocytes supplemented with 10% EBS and lowest with estradiol-17β @1µg/ml. The statistical analysis revealed that CMR was significantly ($p \leq 0.05$) higher in the groups supplemented with 5% EBS and 10% EBS as compared to others. It can be concluded that the supplementation of EBS to IVM medium has positive effect on cytoplasmic maturation rate. Overall results suggest that serum gonadotrophin is less effective than EBS but more effective than estradiol-17β and BFF. Buffalo follicular fluid is more effective than estradiol-17β but less effective than EBS and serum gonodotrophin. Estrodiol-17β is the least effective supplement to increase *in vitro* cytoplasmic maturation rate of buffalo oocytes.

Introduction

The buffalo forms the backbone of India's dairy industry and is considered as the 'bearer cheque' of the rural flock and India's milking machine (Balain, 1999). As per the 19th livestock census India has around 108.7 million of buffalo population and constitutes around 21.23% of total livestock population. Buffalo population of Karnataka is 33.06 lakhs. Buffalo contributes 51% (132430 tonnes) of total nation's milk production and around 1103.85 tonnes of meat production of total nation's meat production(Department of Animal Husbandry Dairying and Fisheries, ministry of agriculture, New Delhi,2014).

The buffalo is the predominant domestic animal for milk and meat production. On average, buffalo is four times as productive as an average indigenous cow in India. India has world's best buffalo dairy breeds and provides superior buffalo germplasm to several countries of the world (Kaikini, 1992).

The domestic water buffalo plays a key role in milk production in many Asian countries including India, and they are able to withstand the adverse environmental conditions of the tropics. However the buffalo are traditionally considered to have a low reproductive efficiency (Pankaj, 2015). The low reproductive efficiency in female buffalo can be attributed to delayed puberty, higher age at calving, long postpartum anoestrus period, long calving interval, lack of overt sign of heat, and low conception rate. In addition, female buffaloes have few primordial follicles and a high rate of follicular atresia.

These factors are responsible for driving a large number of buffaloes to slaughter house either prior to maturity or after calving, which has resulted in enormous loss of genetic resources and significant decline in their number in recent years. Thus there is a need to improve reproductive performances of buffaloes.

In vitro maturation (IVM) of oocytes and *in vitro* fertilization (IVF), *in vitro* culture of embryos and embryo transfer technology appear to be useful techniques for the improvement of reproductive efficiency of buffaloes (Uma, 1997).Embryo transfer technology has made rapid strides in dairy cattle industry and has become viable commercially in some of advanced countries. Therefore, the emphasis has now shifted to *in vitro* embryo production (IVEP)as it can salvage the genetic potential from infertile female and can yield large number of embryos (Kumar and Anand, 2012).

Ovaries of the slaughtered animals are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) (Agrawal *et al.*, 1995).

In vitro maturation (IVM) is the one of the essential step and starting point for lot of biotechnological applications in animals like *in vitro* fertilization (IVF), cloning, transgenic animal production and embryonic stem cell research. Oocytes maturation is the process of complex changes in the protein phosphorylation which transform the primary oocytes in to mature secondary oocytes. Maturation of the oocytes included two aspects viz., nuclear and cytoplasmic maturation.The nucleus and the cytoplasm of the oocytes undergo many changes during maturation, making it receptive to fertilization and competent to support embryonic development (Tomek *et al.*, 2002).

Several workers have studied different aspects of IVM in mammalian oocytes. In most of the studies revealed that media designed originally for cell culture, are not capable of supporting high levels of oocytes maturation and, therefore, are commonly supplemented with sera and hormones, growth factors and antioxidants to improve the maturation rates at high level (Kumar and Anand, 2012). The maturation medium with the selection of protein supplements, hormones for IVM play an important role in subsequent IVF and *in vitro* development.

Many factors and regulatory molecules have been shown either to inhibit or to promote cytoplasmic maturation, either directly or via the cumulus cells. Hormonal conditioning of oocytes at the time of *in vitro* maturation is of paramount importance in achieving cytoplasmic maturation, which is necessary for preparing the female gamete to initiate

normal development through fertilization (Goto *et al.,* 1988). The oocytes maturation process involves the activation and inhibition of enzymes, hormones and growth factors, which results in nuclear and cytoplasmic maturation (Gilchrist *et al.,* 2007). Hormones like luteinizing hormone (LH), follicle stimulating hormone (FSH), estrogens are frequently used as additives in maturation media to enhance the quality of the maturation process.

Some studies have documented that maturation medium supplemented with FF provides appropriate environment to bovine oocytes development (Romero-Arredondo and Seidel, 1996), since it increases the degree of cumulus cells expansion (Aguilar *et al.,* 2001) and enhances the embryonic development (Algriany *et al.,* 2004).

Supplementation of serum in media had favourable effect on maturation. The serum contains a number of known growth factors that have an important role in the regulation of oocyte maturation, particularly via cumulus cells, it also prevents the hardening of the zona pellucida; moreover, the beneficial action of serum may be due to its antioxidant properties (Mahmoud and Nawito, 2003). Estrus buffalo serum (EBS), a rich source of hormones and growth factors, could be used to improve the developmental competence of buffalo oocytes culture *in vitro* (Abid *et al.,* 2008).

An attempt to induce *in vitro* maturation can only be successful in conditions that are similar to those existing *in vivo*. Therefore, the biochemical composition of the oocyte culture media has great influence on the success rate of *in vitro* maturation.

Since, under *in vivo* conditions the oocytes maturation is influenced by various steroid and gonadotropic hormones, it is necessary to supplement the steroid and hormones to the medium in order to sustain the growth and development of oocytes either by directly or through the hormone rich serum medium. Therefore the development of a suitable culture system and components of maturation media for *in vitro* maturation of oocytes is a major component of the *in vitro* embryo production procedures.

Perusal of literature indicates less number of works on optimizing the *in vitro* maturation of buffalo oocytes as compared to the other species. Considering all these points present study was designed to investigate the effect serum gonadotrophin, estradiol-17β, estrous buffalo serum (EBS) and buffalo follicular fluid (BFF) supplementation on *in vitro* cytoplasmic maturation rate.

Materials and Methods

Media, chemical and plastic wares

All the media and chemicals were procured from Himedia Laboratories, Mumbai unless otherwise stated. Folligon® (Pregnant Mare Serum Gonadotrophin, 1000IU/vial) and Chorulon® (Human Chorionic Gonadotrophin, 1500IU/vial) were purchased from Intervet International B.V., Boxmeer, Netherland. The disposable plastic wares used in this experiment were obtained either from Tarsons Products Private Limited, Kolkota or from Himedia Laboratories, Mumbai.

All the media were prepared by using sterile tissue culture grade water. All the working solutions/media excluding OCM were kept for at least 3-4hr in CO_2 incubator at 38°C, 5%CO_2 and 95 % relative humidity for quenching before use. Prepared OCM was kept in BOD at 37°C. The stocks of media were stored at 4°C and used within one month.

Sterilization procedures

Sterilization of all media was done by filtering through 0.2µm syringe driven filters. The glassware and micropipette tips were sterilized by autoclaving at 121ºC for 30min. Fresh sterilized and disposable culture bottles, petridishes, tubes and syringe were used every time. All the equipments were exposed to UV light for 15 minutes before use. In order to avoid bacterial and fungal contamination all the procedure except the aspiration of oocytes, starting from oocyte searching to *in vitro* culture works and media preparation were carried out in highly sterile condition under laminar flow cabinet.

Preparation of buffalo serum and estrous buffalo serum (EBS)

The blood was collected from jugular vein of normal cycling buffaloes that are not in estrus as well as from the buffaloes that are in estrus for the separation of buffalo serum and estrus buffalo serum respectively. The collected blood was kept in slant position and allowed to clot; later blood was centrifuged at 3000rpm for 15min for serum separation. The separated serum was heat inactivated at 56°C for 30minutes, filtered through 0.45µm filter and stored at -20°C in 2.0ml micro centrifuge tubes as aliquots for future use. Same batch of pooled serum was used for all the trials. The buffalo serum was used in the preparation of working oocyte collection medium (OCM) whereas EBS was used as supplement in T5 and T6 groups.

Collection of buffalo follicular fluid (BFF)

Ovaries were collected immediately after slaughter of buffaloes of unknown reproductive status at the local abattoir. The ovaries were maintained in a thermos flask containing warm (35-37°C) normal saline (0.9% NaCl) fortified with 50µg/ml gentamycin sulphate (Gentalab). The ovaries were transported to the laboratory within 2 hrs after slaughter of animals. At the laboratory, the buffalo ovaries obtained from the abattoir were rinsed thoroughly with fresh sterile normal saline supplemented with gentamycin @50µg/ml 5-6 times and final wash was done with Phosphate buffered saline (PBS).

Follicular fluid was aspirated from non atretic, surface follicles (>3mm diameter) of the ovaries using 5ml syringe attached with 18-gauge needle. The pooled follicular fluid was allowed to settle for 10mins and the supernatant was collected. The collected follicular fluid was sterilized by filtering through 0.22µm syringe driven filter and stored in sterile micro centrifuge tubes of 2.0ml capacity at -20°C for subsequent use for IVM as supplement in T7 and T8 groups.

Collection of ovaries and semen sample

Buffalo ovaries were collected immediately after slaughter of the animals of unknown reproductive status at the local abattoir. The ovaries were maintained in a thermos flask containing warm (35-37°C) normal saline (0.9% NaCl) fortified with 50µg/ml gentamycin sulphate (Gentalab).

The ovaries were transported to the laboratory within 2 hrs after slaughter of animals. The frozen buffalo bull semen straws supplied by Department of Animal Husbandry and Veterinary Services, Government of Karnataka to the Department of Veterinary Gynaecology and Obstetrics, Veterinary College, Bidar were utilized for *in vitro* fertilization.

Oocytes collection, processing and grading

Ovaries were collected immediately after slaughter of adult female buffaloes of unknown reproductive status at the local

abattoir. The ovaries were maintained in a thermos flask containing warm (35-37°C) normal saline (0.9% NaCl) fortified with 50µg/ml gentamycin sulphate. The ovaries were transported to the laboratory within 2hrs after slaughter of animals.

In the laboratory, ovaries were washed 6-7 times in warm normal saline solution (37°C) fortified with antibiotic and final two washings with PBS. Washed ovaries were submerged in PBS solution in sterile beaker and were used for oocyte collection. Oocytes were aspirated from all visible non atretic follicles (2-8mm in diameter) by an 18gauge needle attached to 5ml sterile disposable syringe (Dispovan, India) containing 0.5ml oocyte collection media (OCM) (Appendix-I).

The cumulus oocyte complexes (COC) along with follicular fluid was pooled into 50ml sterile plastic tube containing 1-2ml OCM at 37°C and were allowed to settle for 10minutes. Finally the sediments were taken in large petridish (90mm) and oocytes were searched under zoom stereo microscope (Motic, Germany).

The cumulus oocyte complexes (COC) were isolated, evaluated and graded. Only excellent (>5 layers of cumulus cells and evenly granulated cytoplasm) and good (>3 layers of cumulus cells and evenly granulated cytoplasm) COC were collected and washed several times in OCM followed by maturation media (MM) (Appendix II). The recovery rate of oocyte was calculated by dividing the sum of excellent and good quality oocytes recovered with total number of ovaries aspirated.

Ooctye recovery rate (%)

$$= \frac{\text{Total no of excellent and good quality oocytes}}{\text{Number of ovaries aspirated}} \times 100$$

In vitro maturation of oocytes

Graded oocytes were washed in respective maturation media for 4-5 times. After washing, 15-20 oocytes were cultured in 50µl droplets of respective maturation media in 35mm sterile petridish.

Table.1 Composition and supplements of the maturation media in different groups

Groups	Supplements	Detailed Composition of maturation medium
T1	Serum Gonadotrophin @50 IU/ml	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + serum gonadotrophin @50IU/ml
T2	Serum Gonadotrophin @100 IU/ml	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + serum gonadotrophin @100IU/ml
T3	Estradiol-17β @0.5µg/ml	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + Estradiol-17β @0.5µg/ml
T4	Estradiol-17β @1µg/ml	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + Estradiol-17β @1µg/ml
T5	5% Estrous buffalo serum	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + 5%EBS
T6	10% Estrous buffalo serum	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + 10%EBS
T7	5% Buffalo follicular fluid	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + 5%BFF
T8	10% Buffalo follicular fluid	TCM 199+10% FCS+ 0.3% BSA+ hCG @10IU/ml + 10%BFF

The droplet was covered with warm, nontoxic mineral oil and incubated at 38°C, 5% CO_2, 95% relative humidity for 24hrs in CO_2 incubator (Nuaire, USA). The experiment was repeated 6 times for each group as replicates. The selected oocytes were cultured in TCM 199 medium containing 10%FCS, 0.3% BSA and 10IU/ml of hCG with different supplements as shown in Table No 1.

Assessment of cytoplasmic maturation of oocytes

Assessment of oocyte maturation was done on visual basis as per the degree of cumulus expansion under an inverted microscope (Kobayashi *et al.*, 1994).

Degree 0: No expansion
Degree 1: cumulus cells were non-homogenously spread and clustered cells were still observed.
Degree 2: cumulus cells were homogenously spread and clustered cells were no longer present

Only degree 1 and 2 cumulus expanded oocytes were considered as matured ones and cytoplasmic maturation rate was calculated by dividing total number of degree1 and 2 matured oocytes with total number of oocytes utilised for maturation by particular media and expressed in percent CMR

Cytoplasmic maturation rate (%)

$$= \frac{\text{Number of degree1 and 2 matured oocytes}}{\text{Number of oocytes utilised for maturation}} \times 100$$

Results and Discussion

The effect of different supplements to IVM medium on cytoplasmic maturation rate (%) of buffalo oocytes are presented in Table 3. Cytoplasmic maturation rate was assessed based on degree of cumulus cell expansion as degree 0- No expansion, degree 1- cumulus cells were non-homogenously spread and clustered cells were still observed and degree 2- cumulus cells were homogenously spread and clustered cells were no longer present (Plate 5).

The mean percentage values oocytes showing degree-2 cumulus cell expansion in the maturation medium having different supplements viz., T1(serum gonadotrophin @50 IU/ml), T2 (serum gonadotrophin @100IU/ml), T3 (estradiol-17β @0.5µg/ml), T4 (estradiol-17β @1µg/ml), T5 (5% estrus buffalo serum), T6 (10% estrus buffalo serum), T7 (5% buffalo follicular fluid) and T8 (10% buffalo follicular fluid) were 51.01±1.90, 59.42±2.70, 58.25±2.41, 62.04±3.11, 62.84±1.85, 65.62±1.52, 60.47±1.76 and 59.77±1.59respectively.

Similarly the mean percentage values oocytes showing degree-1 cumulus cell expansion were 25.07±2.15, 24.62±2.08, 20.84±1.87, 14.18±1.89, 23.01±1.80, 21.07±1.81, 19.07±1.69 and 18.41±1.78 for T1, T2, T3, T4, T5, T6, T7 and T8 respectively.

The mean percentage values of oocytes that did not show any expansion of cumulus cells for T1, T2, T3, T4, T5, T6, T7 and T8 groups were 19.03±0.53, 15.96±1.24, 19.88±0.60, 23.79±1.38, 14.15±1.06, 12.20±1.06, 20.47±0.86 and 21.82±1.42 respectively. The maturation of these oocytes was considered as degree-0 as there was no expansion of cumulus cells.

Only the oocytes in which the cumulus cell expansion was degree 2 and degree 1 were considered as matured oocytes and accordingly the sum of oocytes showing degree 1 and degree 2 cumulus cell expansions were considered for calculating cytoplasmic maturation rate. The mean percentage of cytoplasmic maturation rate of

culturable oocytes in maturation media supplemented with serum gonadotrophin @50 and 100IU/ml; estradiol-17β @0.5 and 1µg/ml; 5 and 10% estrus buffalo serum, 5 and 10% buffalo follicular fluid were 80.97±0.53, 84.03±1.34; 78.13±1.42, 76.21±1.38; 85.84±1.00, 87.79±1.06; and 79.53±0.86, 80.36±0.52 respectively (Table 3).

Among the eight experimental groups under study the mean values of cytoplasmic maturation rate was highest in the oocytes cultured in IVM medium supplemented with 10% estrus buffalo serum and the lowest cytoplasmic maturation rate was seen with supplementation of estradiol-17β @1µg/ml. The statistical analysis of the data revealed that cytoplasmic maturation rate was significantly ($p \leq 0.05$) higher in the groups supplemented with 5% estrus buffalo serum and 10% estrus buffalo serum as compared to other groups.

There was no significant ($p \leq 0.05$) difference in the cytoplasmic maturation rate among the groups supplemented with serum gonadotrophin @50IU/ml, serum gonadotrophin @ 100 IU/ml, estradiol-17β @0.5µg/ml, estradiol-17β @1µg/ml, 5% buffalo follicular fluid and 10% buffalo follicular fluid.

The comparison of mean values of cytoplasmic maturation rate between two different concentrations of each supplement suggested that there was no significant ($P \leq 0.05$) difference between serum gonadotrophin @50 IU/ml and @100IU/ml, estradiol-17β @0.5µg/ml and @1µg/ml, 5% and 10% estrus buffalo serum, 5% and 10% buffalo follicular fluid.

In Vitro maturation

Serum gonadotropin

Supplementation of serum gonadotrophin @100IU/ml to IVM medium showed better cytoplasmic maturation rate. The beneficial effects of gonadotrophins could be attributed to the fact that they are the primary regulators of oocyte maturation in mammalian oocytes *in vivo* and one of the functions of its preovulatory surge is to suppress the granulosa cell factor that inhibits meiosis (Umadevi, 1997). It is suggested that the presence of gonadotrophins in the maturation media increases the level of intracellular cAMP, the activity of the hyaluronic acid synthesis enzyme system and induced cumulus expansion complexes.

The results of the present work are in agreement with the findings of several researchers in different mammalian species. Mattioli *et al.,* (1991) concluded from their study that gonadotrophins accelerated and facilitated meiotic progression selectively improved cytoplasmic maturation in porcine oocytes which is required to promote the formation of a female pronucleus.

The cumulus expansion was significantly affected in a dose dependent manner by concentrations of FSH, LH, or a combination of FSH and LH (Choi *et al.,* 2000). Nandi *et al.,* (2003) reported that buffalo cystic follicular fluid supplemented with PMSG possess the ability for induction of maturation of buffalo oocytes and could be successfully tried as buffalo oocyte maturation medium.

Accardo *et al.,* (2004) noted that the sheep oocytes cultured without gonadotrophins had no expansion of the granulosa cells. The addition of FSH and/or LH has been shown to improve the expansion of the granulosa cells.

Beenish *et al.,* (2015) reported that supplementation of rhFSH alone and in combination with E2in TCM-199 had highly significant effect on cumulus expansion in buffalo oocytes. Caprine oocytes matured

with 20IU/ml PMSG had a good cytoplasmic maturation rate that allows normal embryo development up to blastocyst stage (Kouamo and Kharche, 2015)

Estrodiol-17β

The mean values of cytoplasmic maturation rate in treatment groups supplemented with estradiol-17β @ 0.5µg/ml and 1µg/ml concentration were 78.13±1.42 and 76.21±1.38 respectively. The supplementation of estradiol-17β @1µg/ml showed the lowest cytoplasmic maturation rate among all the experimental groups. This inhibitory effect of Estradiol-17β on oocyte maturation is observed by McGaughey (1977) in pigs, Eppig and Koide (1978) and Dianne and Tenney (1980) in mouse. Estradiol-17β inhibited the cAMPphosphordiesterase activity of mouse oocyte in a concentration-dependent manner (Kaji, 1987). Anna *et al.*, (2004) observed that Culturing of murine oocytes with the high dose of estradiol-β delayed follicular growth and also suppressed proliferation of granulosa cells and antrum formation. In contrast Fukui *et al.*, (1982) have reported increased *in vitro* maturation rate of bovine oocytes in the presence of estrodiol in culture medium. Similarly, Zheng *et al.*, (2007) have reported improved oocyte developmental competence in rhesus monkey oocytes when they were cultured in *in vitro* maturation medium supplemented with estradiol.

Table.2 Recovery rate (%) of culturable oocytes from buffalo ovaries by aspiration method

Number of ovaries aspirated	Oocytes recovered			Total number of culturable oocytes recovered (Excellent and Good)	Recovery rate of culturableoocytes (%)
	Excellent	Good	Poor		
866	335 (38.68%)	404 (46.65%)	137 (15.58%)	739	85.33

Estrus buffalo serum

The mean values of cytoplasmic maturation rate in the groups supplemented with estrus buffalo serum @ 5% and 10% concentrations were 85.84±1.00 and 87.79±1.06 respectively. Among the eight experimental groups under study the mean values of cytoplasmic maturation rate was highest in the oocytes cultured in IVM medium supplemented with 10% estrus buffalo serum.

The beneficial effect of supplementation of estrus buffalo serum to IVM medium on oocyte maturation could be attributed to the fact that estrus buffalo serum contains various hormones like FSH, LH and E2. Biological role of estrus serum is to compensate for whatever essential elements are missing from the medium by serving as a reservoir for many of the beneficial components, such as different energy substrates, steroids, amino acids, fatty acids, vitamins and growth factors. Samad *et al.*, (1998) evaluated four types of serum supplements viz., estrus cow serum (ECS), estrus buffalo serum (EBS), pro estrus buffalo serum (PrBS) and post estrus buffalo serum (PtBS) added to TCM-199 for *in vitro* maturation of buffalo follicular oocytes.

They recorded oocytes maturation rate of 80.00, 82.08, 78.77 and 66.23 % respectively and concluded significantly high maturation rate in EBS supplemented group followed by ECS, PrBS and PtBS supplemented groups.

Table.3 The effect of different supplements to IVM medium on cytoplasmic maturation rate (%) of buffalo oocytes based on cumulus cell expansion (Mean±SE)

Groups	No. of replicate	No. of oocytes examined	Degree of cumulus cell expansion			Cytoplasmic maturation rate
			0	1	2	
T1	6	95	19.03±0.53	25.07±2.15	51.01±1.90	80.97±0.53^{cd} (77)
T2	6	94	15.96±1.24	24.62±2.08	59.42±2.70	84.03±1.34^{abc} (79)
T3	6	92	19.88±0.60	20.84±1.87	58.25±2.41	78.13±1.42^{de} (72)
T4	6	92	23.79±1.38	14.18±1.89	62.04±3.11	76.21±1.38^{de} (70)
T5	6	92	14.15±1.06	23.01±1.80	62.84±1.85	85.84±1.00^{ab} (79)
T6	6	89	12.20±1.06	21.07±1.81	65.62±1.52	87.79±1.06^{a} (78)
T7	6	93	20.47±0.86	19.07±1.69	60.47±1.76	79.53±0.86^{cde} (74)
T8	6	90	21.82±1.42	18.41±1.78	59.77±1.59	80.36±0.52^{cde} (72)

a,b,c,d,e: Mean values of cytoplasmic maturation rate with different superscripts differ significantly ($p \leq 0.05$)

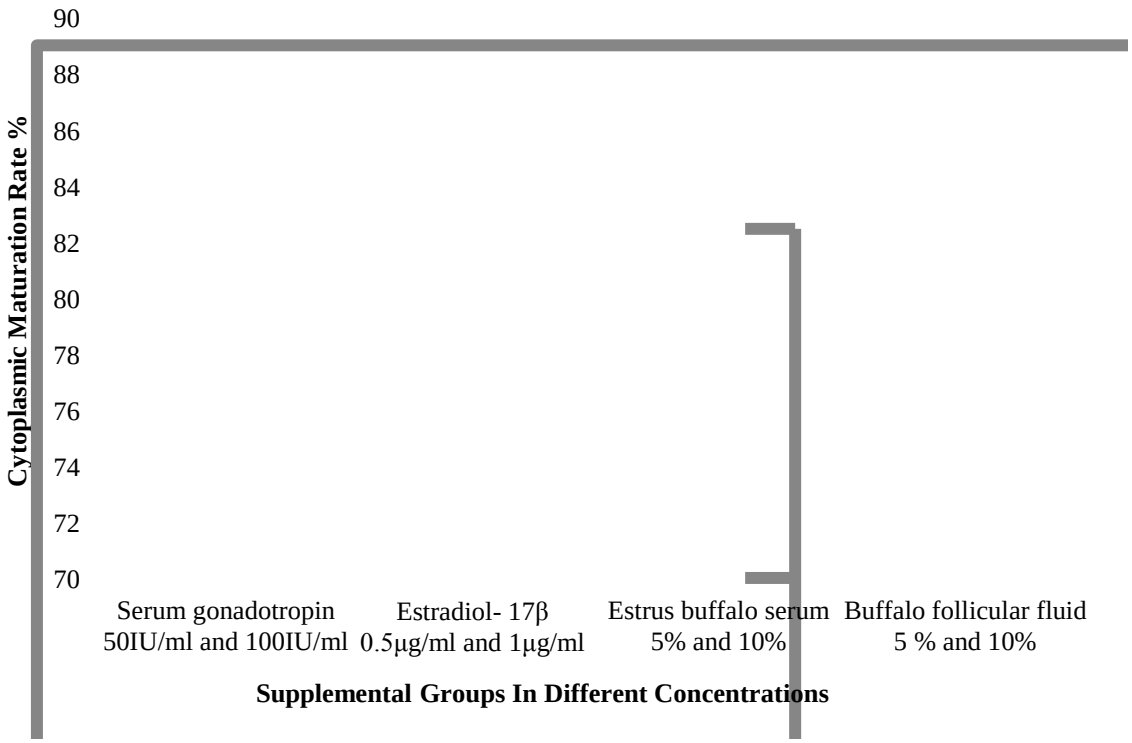

Fig.1 Supplement Groups in different concentrations

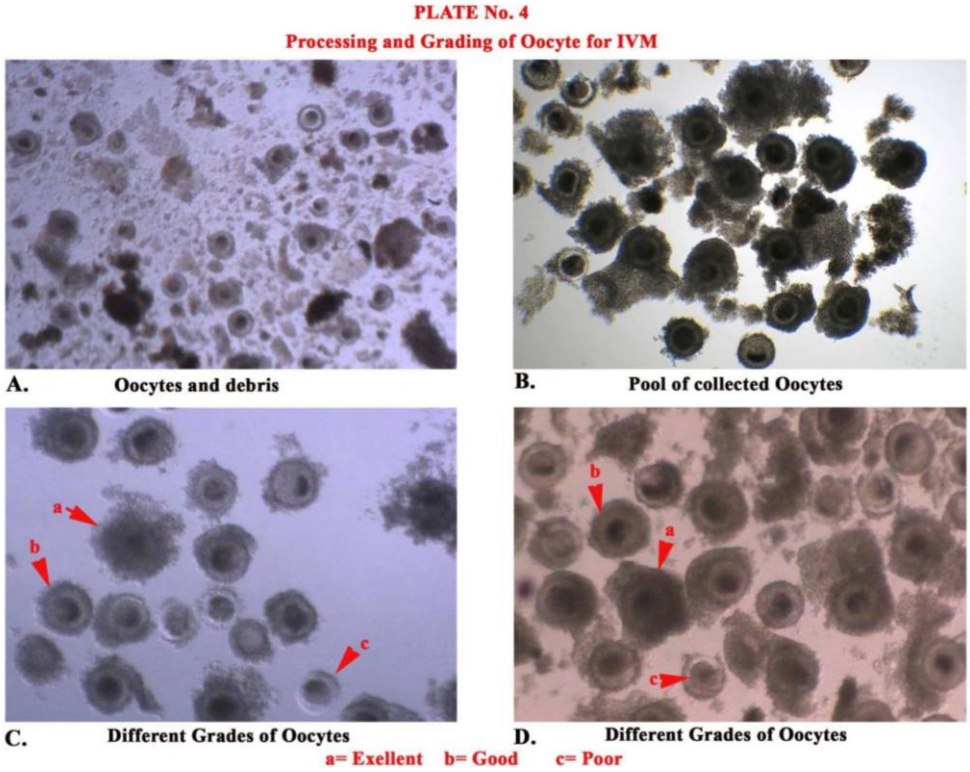

Plate.1 Processing and grading of Oocyte for IVM

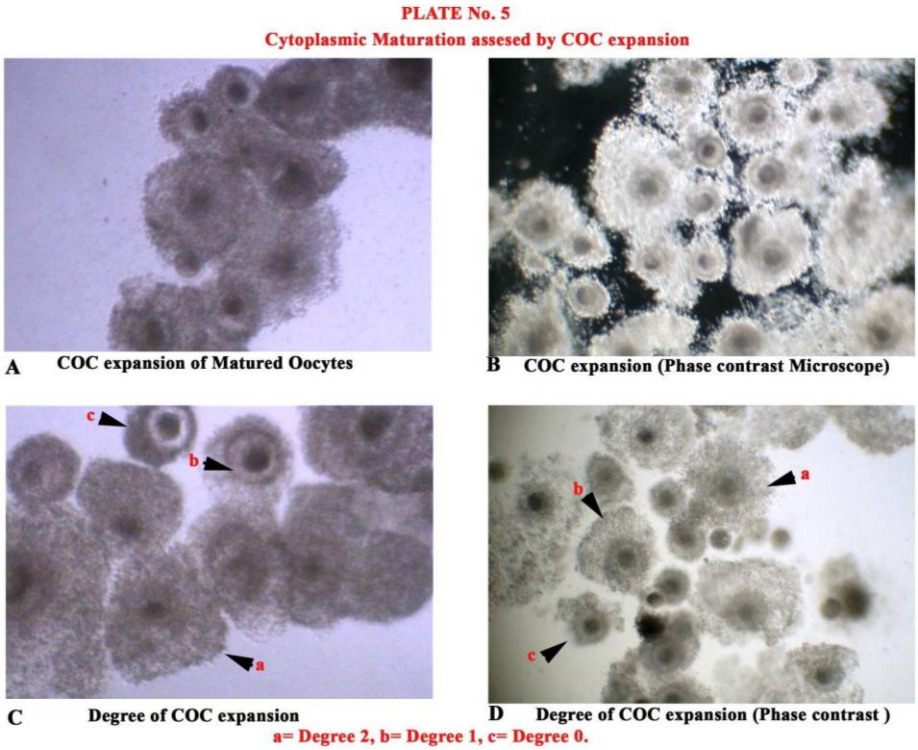

Plate.2 Cytoplasmic Maturation assessed by COC expansion

Suresh and Maurya (2000) observed the maturation rate of buffalo oocytes in TCM-199 containing foetal calf serum, bubaline estrus serum and buffalo calf serum and concluded that the supplementation of bubaline estrus serum can enhance the buffalo oocyte maturation *in vitro*. Anthony *et al.*, (2013) examined the maturation capacity of buffalo, sheep and goat oocytes in the media containing sera of three different groups of buffaloes (regularly cycling, pregnant and repeat breeding). The oocytes maturation rate of buffalo oocytes cultured in media containing sera of the control group and regularly cycling group were not significantly different.

However when oocytes cultured in the media containing sera of pregnant buffaloes the maturation rate were significantly declined. Further significant declined in maturation rate were observed when oocytes cultured in media containing sera of repeat breeding buffaloes.

Buffalo follicular fluid

The mean values of cytoplasmic maturation rate in the groups supplemented with buffalo follicular fluid @ 5% and 10% concentrations were 79.53±0.86 and 80.36±0.52 respectively. The cytoplasmic maturation rate of oocytes supplemented with buffalo follicular fluid was significantly lower than that of estrus buffalo serum supplemented groups.

Mammy *et al.*, (2012) conducted *in vitro* maturation of buffalo oocytes in TCM 199 supplemented with 10% bovine follicular fluid (BFF), 5% bovine serum albumin (BSA) or without supplementation. They concluded that supplementation 10% BFF and 5% BSA significantly increased the maturation rate than the control group. The discrepancy in the results of present experiment with others may be because of variations in the size and maturational status of the follicles from which the follicular fluid was collected.

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