THE EFFECT OF DIFFERENT TYPE OF SERA ON MATURATION RATE OF BUFFALO FOLLICULAR OOCYTES

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ABSTRACT

A total of 1409 follicular oocytes were recovered by slicing method from 456 ovaries of Surti buffaloes collected from the local abattoir. The object was to assess the effect of different categories of buffalo sera (20%, with natural hormones) in relation to BSA (0.6%, Sigma, supplemented with 10 IU/ml of eCG – Folligon and 5 IU/ml of hCG – Chorulon, Intervet) on *in vitro* maturation (IVM) of oocytes in TCM-199 medium. The sera were obtained locally from different categories of animals, viz., fetal bovine serum (FBS, Gibco), neonatal buffalo serum (NBS), oestrus buffalo serum (OBS), post-oestrus buffalo serum (POBS), and anoestrus buffalo serum (AnBS) and processed further for heat inactivation in the laboratory. The oocyte recovery rate was 3.09 per ovary. The average oocyte recovery of grade A, grade B and grade C was 1.02, 1.22 and 0.85, respectively. The maturation rate in presence of POBS, AnBS, FBS, NBS and OBS, each at 20 % level in TCM-199 medium, was found to be 64.63, 54.55, 70.63, 60.48 and 78.16 per cent, respectively, as against 84.00 per cent with 0.6 % BSA supplemented with hormones. The culture medium containing oestrus buffalo serum (OBS) yielded significantly higher ($P<0.05$) maturation rate (at par with BSA) than the other sera used. The highest maturation (90.00 %) of grade A oocytes was found in BSA 0.6 % followed by OBS (86.67 %) and other sera, while in grade B it was in BSA 0.6 % (82.61 %), OBS (81.58%) and FBS (80.00%) – all at par, and for grade C oocytes the higher ($P<0.05$) maturation rate was with BSA 0.6 % (80.56 %) as compared to all sera. According to nuclear maturation, the highest number of oocytes with germinal vesicle was found in medium containing NBS (25.81 %) followed by AnBS (18.18 %) and others. The highest number of GVBD was found in POBS (30.61 %) followed by FBS (29.37 %). The higher number of oocytes with Metaphase-I was in the medium containing BSA 0.6 % (22.13 %) followed by FBS, while, the Metaphase-II stage was found to be higher in medium containing OBS (41.38 %) followed by BSA 0.6 per cent (35.25 %). The degenerated oocytes were lowest in OBS (9.20 %) and highest in AnBS (27.27 %). Overall, the 20 % OBS plain appeared to be an alternate to 0.6% BSA with 10 IU/ml of eCG and 5 IU/ml of hCG for IVM of buffalo follicular oocytes.

Key words: Buffalo, Oocyte, Abattoir ovaries, Slicing, Different type of sera, *In vitro* maturation

INTRODUCTION

In vitro maturation (IVM) is a back bone step in the *in vitro* embryo production. There is a constant need to emphasize the fact that effective oocyte maturation is the foundation of embryo production. Identifying the factors that influence the IVM is essential to improve the *in vitro* embryo production system in buffalo (Chauhan *et al.*, 1998). It has been shown that a higher maturation rate could be reached within 24 hrs of culture if the oocytes had a compact cumulus investment (Chia *et al.*, 1995). In addition to the quality of cumulus

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oocyte-complexes, other factors are also responsible for the success of *in vitro* embryo production. Higher cytoplasmic maturation (60-80%) has been achieved by addition of fetal bovine serum (Chauhan *et al.*, 1999), oestrus buffalo serum (Chauhan *et al.*, 1998) and pro-oestrus buffalo serum (Samad *et al.*, 1998) to the IVM media. Addition of luteinizing hormone (LH) to maturation medium (TCM-199 + 20% EBS) improved maturation rate from 47 per cent to up to 77 per cent (Totey *et al.*, 1992). A maturation rate of 70-80 per cent has been achieved by using TCM-199 supplemented with FBS and 5 mg/ml FSH-p (Chauhan *et al.*, 1999). Since the presence of serum macromolecules is necessary for culture of oocytes, an attempt was made to study the effect of supplementation of plain FBS, BSA, OBS, AnBS and POBS in relation to BSA with eCG and hCG in TCM-199 medium for *in vitro* maturation of buffalo follicular oocytes.

MATERIALS AND METHODS

This study was carried out at Department of Animal Biotechnology, College of Veterinary Sciences, AAU, Anand, over a period of 6 months.

Collection and Slicing of Ovaries: Ovaries (456) of the matured Surti buffaloes slaughtered at a local abattoir of Anand in presence of one of the authors were collected and transported to the laboratory within one hour in a flask containing normal saline (0.9 % NaCl) at PH 7.0 supplemented with 50 µg/ml Gentamicin (Sigma, G 3632). The extraneous tissues and fat were removed in the laboratory and ovaries were washed with 70 per cent alcohol to check contamination, followed by three washes of the normal saline (39°C). The washed ovaries were sliced with a BP blade and transferred in to 100 mm disposable petri-dish (Tarson® INDIA) with warm normal saline. The contents of all sliced follicles were examined for cumulus oophorus complexes (COCs) and oocytes by using stereoscopic microscope (Nikon SMZ-2B, Tokyo, Japan). They were transferred to pre-warmed drops of 100 µl of gonadotropin free Hepes buffered TCM-199 medium, which was covered with 3 ml of silicon oil (Sigma) in the 35 x 10 mm (Tarson® INDIA) plastic petri-dish. The COCs were classified according to Leibfried and First (1979). The presence of cumulus cells surrounding the immature oocytes was a pre-requisite for successful *in vitro* maturation of buffalo oocytes.

Sources and Collection of Sera: Collection of blood and separation of serum was done as per Lu *et al.* (1987). Buffaloes of different physiological status, viz., oestrus, post-oestrus, anoestrus and neonate, were used as sources of different categories of sera. All the sera samples were inactivated by heating at 56°C for 30 min in water bath, filtered through a 0.22 mm syringe filter and stored at -20°C until used.

All these sera differ in their levels of biochemical, hormonal and ion concentration according to the stage of reproductive cycle when harvested. Hence, the object of using these sera simultaneously was to check which of the serum constituents inhibits or supports the IVM of buffalo oocytes and if any one of them can be used as a substitute of BSA.

In vitro Maturation of Buffalo Oocytes: For *in vitro* maturation of COCs, TCM-199 medium was used as the basic medium to which different sera @ 20 % (plain with natural hormones) and BSA 0.6 % (supplemented with 10 IU/ml of eCG – Folligon and 5 IU/ml of hCG – Chorulon, Intervet) were added to see their effect on maturation rate. The pH of all culture media was maintained at 7.4. The media were sterilized by filtration through 0.22 µm Millipore filter. Droplets of 200 µl of maturation media were prepared in a petri-dish covered with mineral oil and equilibrated for half an hour with 5 per cent CO_2 and 95 per cent humidity.

Selected oocytes were washed three times in 10 drops of fresh pre-warmed TCM-Air medium and subjected to final washing in 10 drops of maturation medium of TCM-Air before being transferred to the maturation droplets. About 10-20 oocytes were transferred to the droplets in TCM-199 in different batches containing different sera like OBS, AnBS, POBS, NBS, FBS all at 20 per cent level plain as against 0.6 % concentration of BSA-FAF with hormones. Oocytes were cultured for 24 hrs at 5 per cent CO_2,

39°C temperature and 95 per cent humidity in an incubator.

Assessment of Nuclear Maturation: Maturation of oocytes was assessed on the basis of the expansion (gross) and dispersion of cumulus cells surrounding the oocytes reaching at Metaphase-II with the extrusion of one polar body. To assess the nuclear maturation rate, randomly selected oocytes were stained with Hoechst 33342 stain. The maturation of oocytes was assessed under the inverted microscope with florescence unit (Leica DMIL, Germany). The percentages of oocytes showing various maturational changes in medium containing different sera or BSA were worked out and were analyzed statistically using chi-square test (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The different quality of oocytes were cultured in FBS, NBS, POBS, AnBS and OBS, all natural at 20 per cent level, to study their effect on *in vitro* maturation of buffalo oocytes in relation to standard BSA (0.6 %) with 10 IU/ml of eCG and 5 IU/ml of hCG that gave the best result in the preliminary trial. The results are summarized in Table 1.

Table 1 : Effect of different types of sera on *in vitro* maturation of Surti buffalo oocytes in relation to 0.6 % BSA

Media	No. of oocytes		
	Examined	Matured	Maturation rate (%)
BSA 0.6	122	103	84.43^{a}
POBS	147	95	64.63^{b}
AnBS	154	84	54.55^{c}
FBS	143	101	70.63^{ab}
NBS	124	75	60.48^{bc}
OBS	174	136	78.16^{a}

Values bearing different superscripts within the column differ significantly (P<0.05).

In this study, OBS was found superior to other types of sera, although it was still inferior to BSA 0.6 per cent. Statistically, the maturation rate of oocytes in OBS differed significantly (P<0.05) from that in POBS, AnBS and NBS. The BSA 0.6 per cent yielded higher maturation rate, but it was not statistically different from OBS. The higher maturation rate of buffalo oocytes obtained in OBS in this study is in agreement with Kim *et al.* (1990), Samad *et al.* (1998) and Jamil *et al.* (2007). Using different media and concentrations of OBS with and without hormones, Totey *et al.* (1992) found maturation rate of 69.2 to 81.7 per cent, while Madan *et al.* (1994^{a}) obtained it as 76 to 84 per cent. Madan *et al.* (1994^{b}) obtained good result in 10 per cent OBS than 5 per cent concentration. Totey *et al.* (1992) and Chauhan *et al.* (1996) showed that 20 per cent concentration of OBS provides higher maturation rate of oocytes. Moreover, Singh *et al.* (1989) and Kito and Bavister (1997) reported that the effects of gonadotropins on nuclear maturation, cumulus expansion and oocyte morphology are modulated by serum. In our study, we observed that there was no significant difference in maturation rate for commercial FBS and prepared NBS, which was in agreement with the findings of Mahmoud and Nawito (2005). According to Ocano-Quero *et al.* (1999) LH in oestrus serum initiates resumption of meiosis in dictyate oocytes, which are arrested in Metaphase-II stage. The superior results found with OBS than other sera in our study could be due to its high content of gonadotrophins, estrogens and growth factors that have important roles in regulation of oocyte maturation, particularly via cumulus cells (Skinner, 1990). The highest maturation rate observed with BSA in our study could be due to supplementation of eCG and hCG in that medium as compared to other sera.

The maturation rate of oocytes in NBS in this study was lower than that found by Singh *et al.* (1989) and higher than that reported by Jainudeen *et al.* (1993) using FBS. Totey *et al.* (1993) obtained a nuclear maturation rate of 47 per cent with TCM-199 + 20 per cent OBS, which was improved with addition of LH up to 77 per cent (Totey *et al.*, 1992), while Madan *et al.* (1994^{b}) reported nuclear maturation rate of around 80 per cent in TCM-199 + 20 per cent OBS without the use of any hormone. Our results coincided to their

findings. Umadevi and Reddy (1998) found significantly higher maturation rate in media containing pro-oestrus serum than oestrus serum. The higher maturation rate of buffalo oocytes in BSA than OBS in the present study is in agreement with the result reported by Chauhan *et al.* (1996). Further, Chauhan *et al.* (1998) noted that media containing superovulated buffalo serum (10 %) provided higher maturation rate than the media containing oestrus buffalo serum, FBS and steer serum. The addition of 20 per cent OBS with gonadotrophins and oestradiol in TCM-199 or Ham's F-12 resulted in better maturation rate of buffalo follicular oocytes than medium containing only OBS (Chauhan *et al.*, 1996; Abdoon, 2001). All these reports indirectly supported our finding of significantly reduced maturation rate of oocytes found in AnBS, as it contains lowest levels of

Table 2 : Assessment of maturation of buffalo follicular oocytes in TCM-199 medium with different sera based on nuclear stages

Sources of Serum	Nuclear stage				
	Germinal vesicle (%)	Germinal vesicle breakdown (%)	Metaphase- I (%)	Metaphase - II (%)	Degenerated oocytes(%)
BSA 0.6	4.92^{b}(6/122)	27.04^{a}(33/122)	22.13^{a}(27/122)	35.25^{ab}(43/122)	10.66^{b}(13/122)
POBS	14.97^{ab}(22/147)	30.61^{a}(45/147)	13.60^{b}(20/147)	20.41^{bc}(30/147)	20.41^{a}(30/147)
AnBS	18.18^{a}(28/154)	24.68^{ab}(38/154)	12.34^{b}(19/154)	17.53^{c}(27/154)	27.27^{a}(45/154)
FBS	9.79^{b}(14/143)	29.37^{a}(42/143)	15.38^{ab}(22/143)	25.88^{bc}(37/143)	19.58^{a}(28/143)
NBS	25.81^{a}(32/124)	20.97^{b}(26/124)	11.28^{b}(14/124)	28.23^{bc}(35/124)	13.71^{ab}(17/124)
OBS	12.64^{ab}(22/174)	21.84^{b}(38/174)	14.94^{ab}(26/174)	41.38^{a}(72/174)	9.20^{b}(16/174)

Values bearing different superscripts within the column differ significantly (P<0.05).

proteins, minerals and even steroid and glycoprotein hormones. Use of TCM-199 as basic medium along with different categories of sera gave better maturation rate than other basic media like Ham's F-10 (Raza *et al.*, 2001).

Nuclear Maturation of Oocytes: The results of nuclear maturation, as revealed by Hoescht 33342 staining of oocytes showing cytoplasmic changes, are presented in Table 2, and are also illustrated in Plate 1.

The highest percentage of Metaphase-II was observed in OBS (41.38) followed by BSA 0.6 per cent (35.25), NBS (28.23), FBS (25.88), POBS (20.41), and AnBS (17.53) with corresponding decrease in percentages of degenerated oocytes. The percentage of oocytes with germinal vesicle was significantly (P<0.05) higher in NBS than FBS (Table 3). Use of different sera and BSA along with basic medium TCM-199 significantly (P<0.05) affected the proportion of oocytes that reached Metaphase-II probably because of variable amount of hormones and other catalysts present in them. Similar results were obtained by Bavister *et al.* (1992). Our findings of cytoplasmic maturation are in agreement with the report of Boni *et al.* (1992).

The highest maturation of grade A oocytes was found in BSA 0.6 per cent (90.00 %) followed by OBS (86.67 %) and other sera. While in grade B oocytes the highest maturation was with BSA 0.6, OBS and FBS (all at par), and for grade C oocytes, BSA 0.6 per cent was the best (80.56 %) and it differed significantly (P<0.05) from all sources of sera, which were at par. The discrepancy in the results obtained in different studies may be due to different selection criteria used for categorizing oocytes, difference in the culture conditions (Motlik and Fulka, 1986), source of sera (Younis *et al.*, 1989) and the seasons during which the

oocytes were collected and used for *in vitro* maturation (Selvaraj *et al.*, 1992), as these factors have been reported to influence the rate of maturation of oocytes *in vitro*.

Thus overall, it may be concluded that the 20 % OBS plain can be an alternate to 0.6% BSA supplemented with 10 IU/ml of eCG and 5 IU/ml of hCG for IVM of buffalo follicular oocytes.

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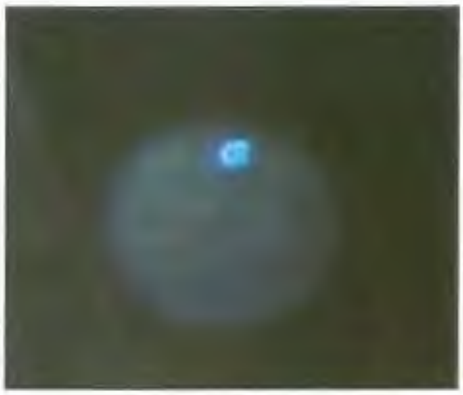
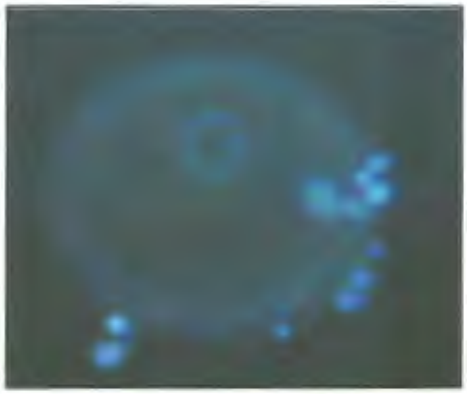
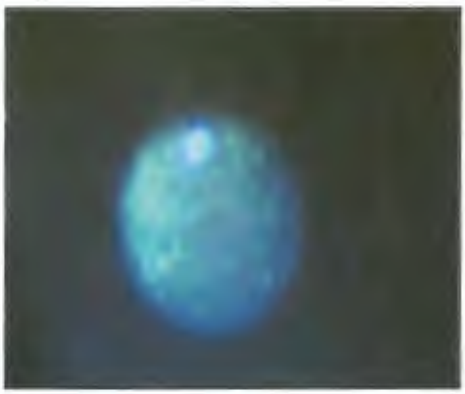


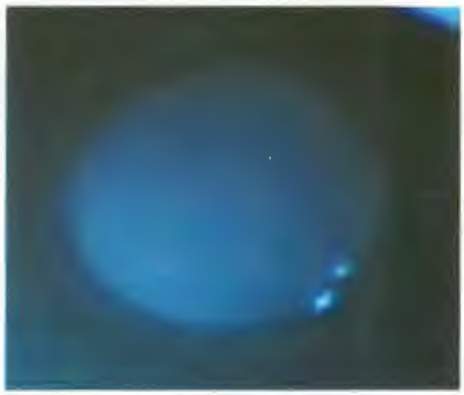
A. Germinal Vesicle (GV)



B. Germinal Vesicle Break Down (GVBD)



C. Metaphase - I (M-I)



D. Metaphase - II (M-II)

Plate 1: In-Vitro Matured Buffalo Oocyte with Hoechst 33342 stain

Seminar

The West Bengal chapter of ISSAR organised a seminar on Innovative approaches to augment fertility in bovines on 11.9.2009 at Kolkatta. Dr. Shiv Prasad, Professor, Department of Animal Reproduction, Gynaecology and Obstetrics College of Veterinary Science, Pantnagar delivered a guest lecture during the seminar.