

Effect of Media, Sera and Hormones on *in vitro* Maturation and Fertilization of Water Buffalos (*Bubalus bubalis*)

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Abstract: This study was carried out to investigate the effect of two media and adding supplements or hormones to IVM medium on buffalo oocyte maturation rate. Oocytes were cultured either in TCM-199 or Ham's F-10 with BSA or FBS with or without hormonal supplements (PMSG, FSH and E₂) or βME, under defined conditions (38°C, 5% CO₂, high humidity) for 22-24 h. The matured oocytes were fertilized *in vitro* by frozen thawed semen capacitated in either Brackett-Oliphant or TALP media. Maturation rate was not affected by media type, but was affected by the supplements added to the media. The maturation rates were 75.0±3.1, 68.0±2.4, 80.4±4.4, 73.8±3.4 and 67.5±3.1% for TCM-199 supplemented with FBS, BSA, FBS+PMSG, FBS+FSH and FBS+E₂, respectively) and 78.4±3.2 and 66.7±2.6% for oocytes cultured in Ham's F-10 supplement with FBS and BSA, respectively. βME added to TCM-199 yielded a higher maturation (95.2±2.9%, p<0.05). Using FBS gave better maturation than BSA, while PMSG, FSH or E₂ had no effect on maturation rate. It is concluded that buffalo oocytes can be matured in TCM or Ham's F-10 supplemented with FBS with best results obtained when βME is added to the media. TALP gave higher fertilization results (33.7±2.3%) compared to BO (15.5±1.2%) (p<0.05).

Key words: Buffalo, oocyte, IVM, IVF, media, hormones

INTRODUCTION

Limited research has been done with *in vitro* fertilization in buffalos compared to cattle and other species (Gasparrini, 2002). Although, successful pregnancies have resulted, efforts to improve the *in vitro* development rates are still needed. Comparison of media types and supplements to the media for oocyte maturation, fertilization and embryo culture have yielded varying results (Totey *et al.*, 1993; Chauhan *et al.*, 1997; Ravindranatha *et al.*, 2003; Gasparrini *et al.*, 2006). The present study deals with two experiments on factors that can influence *in vitro* success rate *in vitro* maturation media and fertilization media. Maturation rate was compared using two commonly used media (TCM-199 and Ham's F-10) supplemented with Fetal Bovine Serum (FBS) or Bovine Serum Albumin (BSA). Three hormonal treatments, Follicle Stimulating Hormone (FSH), Pregnant Mare Serum Gonadotropin (PMSG) and Estradiol (E₂) as well as the chemical antioxidant β-Mercaptoethanol (βME) were tested with TCM-199 + FBS. The second trial compared fertilization success and cleavage rates between

two fertilization media (Brackett and Oliphant (BO) and Tyrodes Albumin Lactate Pyruvate (TALP) medium) of buffalo oocytes matured in either TCM-199 or Ham's F-10.

MATERIALS AND METHODS

Ovary collection: Ovaries from apparently normal reproductive organs of adult Egyptian buffalos of unknown breeding history slaughtered in El-Monieb (Giza) and El-Warak abattoirs were collected within 20 min after slaughter and kept in a Thermos containing physiological saline (0.9% NaCl with 100 µg mL⁻¹ Streptomycin and 100 i.u. mL⁻¹ Penicillin) at 32-35°C. All reagents were obtained from Sigma unless otherwise stated. The ovaries were transported to the laboratory within 3 h of slaughter (Lee and Fukui, 1995). The ovaries were dissected from surrounding tissues and rinsed in 70% ethanol for few minutes to eliminate surface organisms. This was followed by washing several times in normal saline at 37°C, until obtaining clear saline free from blood and then kept in water bath at 37°C during oocyte collection.

Harvesting of oocytes: Oocytes were aspirated from vesicular follicles (2-8 mm in diameter) using an 18-gauge needle attached to a 10 mL disposable syringe containing 1 mL of aspiration medium (Modified Phosphate-Buffered Saline (MPBS) with 5% (v v⁻¹) heat inactivated FBS). The follicular fluid containing oocytes was pooled into a sterile 50 mL plastic Falcon tube (Falcon, USA) and allowed to settle for 30 min in a water bath at 37°C. After settling, about 5 mL of the sediment was aspirated and placed in 10 cm diameter polystyrene sterile petri dish (Nunclon, Denmark).

Oocytes were picked up with a sterile glass pipette under a stereomicroscope and transferred into another dish containing MPBS. The oocytes were classified into different grades according to the condition of surrounding cumulus cells and cytoplasm of the oocyte (Leibfried and First, 1979). Oocytes with compact multi-layered cumulus investment and evenly granulated cytoplasm (Grade 1 as described by Chauhan *et al.* (1998) were selected for *in vitro* maturation.

The selected oocytes were washed three times in maturation medium (pH 7.4) before the *in vitro* maturation droplets were pooled. The oocytes (10-20 oocytes) were placed in 50-100 μ L droplet of maturation medium in a four-well culture plate (Nunclon, Denmark) and covered with sterilized mineral oil (Sigma, USA). The culture dishes were placed in a CO₂ incubator (95% relative humidity, 5% CO₂ at 38°C) for 22-24 h.

Experimental design: The freshly collected good quality buffalo oocytes were divided according to the type of maturation media into two groups: Group I: TCM-199 medium and Group II: Ham's F-10 medium. All media used contained antibiotic (50 μ g mL⁻¹ gentamycin).

Each of the two media groups were subdivided into the following subgroups based on supplement added: 10% FBS (subgroup 1), 3 g L⁻¹ BSA (subgroup 2). In addition, oocytes were matured in TCM-199 plus 10% FBS plus 50 μ g mL⁻¹ PMSG (subgroup 3), 10% FBS plus 50 μ g mL⁻¹ FSH (subgroup 4), 10% FBS plus 1 mg mL⁻¹ E₂ (subgroup 5), 10 μ M mL⁻¹ β -Mercaptoethanol (β ME) added to TCM-199 (subgroup 6).

After 24 h maturation rate was assessed according to the degree of cumulus cells expansion into one of four grades as described by Hunter and Moor (1987).

Sperm capacitation: Spermatozoa were capacitated using either Bracket and Oliphant medium (BO) (Bracket and Oliphant, 1975) or TALP medium. Frozen semen from multiple buffalo bulls stored in 0.25 mL straws were thawed in water bath at 37°C for 1 min. Semen was obtained from the collection of the Division of Animal

Reproduction and Artificial Insemination of the National Research Center (Dokki, Egypt). One bull was used per run so each bull was used across all treatments.

For sperm capacitated in BO medium spermatozoa were washed twice at 2500 rpm for 5 min using semen washing solution of BO medium (Bracket and Oliphant, 1975) supplemented with 20 μ g mL⁻¹ heparin plus 3.383 mg mL⁻¹ caffeine (Niwa and Ohgoda, 1988). Then spermatozoa were maintained in an incubator at 37°C, 5% CO₂ for 1 h for capacitation (Irritani and Niwa, 1997). The spermatozoa were re-suspended in 1 mL of semen diluents solution of BO medium and the sperm number was counted using a hemocytometer and adjusted to be 2 \times 10⁶ mL⁻¹.

For sperm capacitated in TALP, frozen-thawed buffalo semen was added to tubes containing 4 mL of Sperm-TALP medium (SP-TALP) and left in a water bath (37°C) for 1 h for swim up (Parrish *et al.*, 1986). The upper layer of four tubes was removed using a plastic Pasteur pipette and centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the sperm pellet was re-suspended in 5 mL SP-TALP solution and centrifuged at 2500 rpm for 5 min.

After removal of the supernatant, spermatozoa were re-suspended in 1 mL Fert-TALP (F-TALP base stock, supplemented with 6 mg mL⁻¹ BSA plus 0.25 μ M pyruvate and 2 μ L mL⁻¹ Heparin stock) and sperm number was counted using a hemocytometer. The final sperm concentration was adjusted to be 2 \times 10⁶ mL⁻¹ by adding Fert-TALP medium.

***In vitro* fertilization procedure:** Oocytes matured in either TCM-199 + FBS or Ham's F-10 + FBS were partially denuded from the surrounding cumulus cells to allow easy penetration of the sperm cells. They were washed twice in pre-warmed IVF medium to maintain the defined component of the IVF medium. *In vitro* matured oocytes were *in vitro* fertilized in either BO or TALP medium.

For BO matured oocytes were washed twice in oocyte washing solution of BO medium supplemented with 10 mg mL⁻¹ of BSA. A total of 15-20 matured oocytes were placed in each well of the culture dish containing 50 μ L of fertilization medium to which 75 μ L of sperm suspension was added. A layer of 200 μ L of sterile liquid paraffin oil was placed to cover the sperm-oocyte mixture then incubated for 5 h at 38.5°C under 5% CO₂ in humidified air (Irritani and Niwa, 1997).

For TALP matured oocytes were washed with HEPES-TALP and partially denuded. About 15-20 matured oocytes were placed in each well of the culture dish containing 50 μ L of fertilization medium to which 75 μ L of sperm suspension was added. A layer of 200 μ L of sterile

liquid paraffin oil was placed to cover the sperm-oocytes mixture then incubated for 20-22 h at 38.5°C under 5% CO₂ in humidified air (Parrish *et al.*, 1986). The cumulus cells that were detached from the cultured oocytes were kept and the same culture droplets were used for the culture of oocytes after *in vitro* fertilization.

The culture medium was replaced with fresh medium (pre-incubated for at least 2 h inside the incubator) prior to culture of fertilized oocytes. After the sperm-oocytes co-culture and prior to transfer to the *in vitro* culture droplets, oocytes were washed four times in a washing medium (culture medium). Nine and one-half milliliter of TCM-199 was supplemented with 5% FBS + 50 µg mL⁻¹ gentamycin were used for removal of sperm cells attached to or surrounding the zona pellucida. Cleavage rate was checked at 24 h for evidence of fertilization.

Statistical analysis: The proportions of oocytes that exhibited maturation or fertilization were arcsine transformed and analyzed by Analysis of Variance (ANOVA) using the GLM procedure of SAS for Windows 9.1 (SAS, Cary, NC) (Scheffler, 1979). Differences were to be considered significant at p<0.05.

RESULTS AND DISCUSSION

In vitro maturation of buffalo oocytes: There were no differences, when comparing oocyte maturation rates between TCM-199 and Ham’s F-10 with either FBS or BSA as supplements (Table 1). Supplementation with FBS increased maturation rates compared to BSA supplementation for both media (p<0.01). Addition of hormones (FSH, PMSG, or E₂) to TCM-199 with FBS did not increase maturation rate over supplementation with FBS alone. There was a significant (p<0.01) increase in maturation rate of buffalo oocytes matured in TCM-199 plus βME (95.2±2.9%) in comparison to other supplements to TCM-199 (75.0±3.1, 68.0±2.4, 80.4±4.4, 73.8±3.4 and 67.5±3.1%, respectively for FBS, BSA, FBS+PMSG, FBS+FSH and FBS+E₂).

In vitro fertilization of buffalo oocytes: The present results reveal that the *in vitro* fertilization rate of buffalo oocytes matured in TCM-199 + FBS was higher when

capacitation and fertilization was carried out in TALP medium than BO medium (33.7±2.3% vs. 15.5±1.2%) (Table 2). For oocytes matured in Ham’s F-10 + FBS there was no difference between BO and TALP (Table 2).

Supplementation of both TCM-199 and Ham’s F-10 with FBS resulted in improved buffalo oocyte maturation compared with maturation in those media supplemented with BSA. These results agree with those of Liebfried-Rutledge *et al.* (1986) and Wang *et al.* (1997) with bovine and porcine oocytes comparing FBS supplementation of IVM media with BSA supplementation. Serum contains hormones and other uncharacterized factors that can aid in oocyte maturation. Totey *et al.* (1993) found that FBS increased buffalo oocyte maturation rates over media alone. Bovine serum albumin serves as a protein source and may have hormones bound to it therefore media containing BSA is considered semi-defined but is likely to contain fewer uncharacterized factors than FBS (Farin *et al.*, 2001).

Addition of hormones (FSH, PMSG, E₂) to TCM-199 + FBS did not increase maturation rate compared to TCM+FBS without hormones (Table 1). Madan *et al.* (1994) observed that high maturation and fertilization rates and even successful pregnancies can be achieved for buffalo COC using TCM-199 with serum supplementation in the absence of hormones. However, Chauhan *et al.* (1997) reported that buffalo COCs matured in medium with FBS + FSH-P had increased maturation rates compared to FBS without FSH-P. Totey *et al.* (1992; 1993) and Palta and Chauhan (1998) also reported that adding hormones to the maturation media improved buffalo maturation rate.

Table 1: Maturation rate of buffalo oocytes cultured in TCM-199 and Ham’s F-10 and enriched with Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), Pregnant Mare Serum Gonadotropin (PMSG), Follicle Stimulating Hormone (FSH), Estradiol (E₂) or β-Mercaptoethanol (BME). Values in the same column with different superscripts differ at p<0.05

Media + supplement	Total oocytes	Matured oocytes	Maturation rate (%)
TCM+BSA	125	85	68.0±2.4 ¹
TCM+FBS	424	318	75.0±3.1 ²
TCM+FBS+PMSG	56	45	80.4±4.4 ²
TCM+FBS+FSH	263	194	73.8±3.4 ²
TCM+FBS+E ₂	40	27	67.5±3.1 ²
TCM+BME	188	179	95.2±2.9 ³
Hams F-10+BSA	39	26	66.7±2.6 ¹
Hams F-10+FBS	88	69	78.4±3.2 ²

Table 2: Fertilization rate for buffalo oocytes in different maturation media with sperm capacitated in either Bracket-Oliphant (BO) or Tyrodes albumin Lactate Pyruvate (TALP). Values in the same column with different superscripts are significantly different (p<0.001)

Maturation media	TCM-199 + FBS			Ham’s F-10 + FBS		
	No. of oocytes	No. of fertilized oocytes	Fertilization rate (%)	No. of oocytes	No. of fertilized oocytes	Fertilization rate (%)
BO ¹	155	24	15.5±1.2 ^a	51	16	31.3±3.2
TALP ²	83	28	33.7±2.3 ^b	53	22	41.5±3.5

¹Bracket and Oliphant, ²Tyrodes albumin lactate pyruvate

In the present study, maturation rate did not differ between TCM-199 and Ham's F-10 (Table 1). Both media gave similar results when supplemented with FBS or BSA. These results are comparable to those obtained by Hammam *et al.* (1997) who reported that Ham's F-10 could be used as a maturation and culture medium of buffalo oocytes. Totey *et al.* (1992) found that IVM of buffalo COC was more successful with Ham's F-10 + FBS and hormones than TCM-199 with FBS and hormones. The present study did not include Ham's F-10 with hormone supplementation. The differences due to maturation medium might be attributed to the differences in the constituents of these media (Hawk and Wall, 1994).

The highest rate of oocyte maturation was achieved in TCM-199 + BME group (95.2%, Table 1). These results support the findings of Abeydeera *et al.* (1999) and De Matos and Furnus (2000) on the effects of BME on porcine and bovine oocyte maturation, respectively. In buffalo Gasparrini *et al.* (2000, 2003, 2006) found that supplementation with other thiol compounds during IVM improved *in vitro* oocyte maturation in buffalo by increasing cytoplasmic glutathione concentrations. Songsasen and Apimeteetumrong (2002) added BME to TCM-199 with FSH and found improved buffalo oocyte maturation and fertilization rates although, the percent reaching the blastocyst stage was not different. In the present study, BME was able to increase IVM of buffalo oocytes matured in TCM-199 without FBS or FSH. The current findings along with the previous literature suggest that buffalo oocyte maturation media should contain thiol compounds for a high rate of success.

The fertilization medium used for IVF not only affects the proportion of oocytes capable of undergoing fertilization but also their subsequent embryonic development (Madan *et al.*, 1994). The current study found higher fertilization with TALP than with BO. These results differ from those found by Bracket and Oliphant (1975) for rabbits. Totey *et al.* (1992) found that frozen-thawed buffalo spermatozoa prepared in Bracket and Oliphant (BO) medium and treated with 5 mmol mL⁻¹ caffeine plus 10 µg heparin showed a higher fertilization rate (29.8%) than those treated in H-TALP and treated with 10 µg heparin mL⁻¹ (19.6%). Ravindranatha *et al.* (2003) found that cleavage rates were higher when sperm were processed in BO compared with H-TALP medium.

The differing results in this study might be explained by Sirard and Lambert (1985) and Stubbings and Woski (1991), who reported that sperm of individual bulls vary in the frequency of penetration. Moreover, Bracket and Oliphant (1975) indicated that individual variations are present in the fertilizing ability of the ova.

CONCLUSION

Both the types of media used in the present study did not differ in buffalo oocyte maturation with either BSA or FBS as supplements. Supplementation with FBS resulted in a greater percentage of mature oocytes compared with BSA. Addition of hormones did not improve oocyte maturation compared with TCM-199 + FBS alone in the current study. Addition of BME to TCM-199 resulted in higher oocyte maturation rates. Fertilization rates were higher with TALP compared with BO for TCM-199. Multiple media provide satisfactory maturation, fertilization and cleavage rates for buffalo sperm and oocytes.

ACKNOWLEDGEMENTS

This research was supported financially through US-Egypt Binational Research Project: BIO-009 awarded to A.M. Hammam and C.S. Whisnant.

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