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Improving *in vitro* maturation and cleavage rates of buffalo oocytes

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Abstract

The aim of the present study was to improve *in vitro* maturation and cleavage rates of buffalo oocytes. Good quality oocytes were divided into two experiments. In Experiment 1 oocytes were cultured for 24 h in a CO_2 incubator at 38.5°C either in TCM-199, Ham's F-10, MEM or FertiCult medium supplemented with either 10% FCS or 0.3% BSA. Experiment 2 was carried out to investigate the effect of different hormones (either 50 µg/ml eCG, 50 µg/ml FSH or 1 mg/ml E2) added to four of the afore mentioned media enriched with 10% FCS at the same culture conditions. Matured oocytes were fertilized *in vitro* using frozen thawed semen capacitated with heparin and caffeine. The sperm-oocytes were co-cultured for 22 h in BO or TALP medium. The fertilized oocytes were cultured in either BO or TALP medium for an additional five days at the same culture conditions and checked daily for cleavage. Addition of FCS to all media led to a higher maturation rate, without any significant variation, than BSA did (75.6 vs. 71.3%). Although no influence on the maturation rate was observed, addition of eCG to TCM-199, Ham's F-10 or MEM media resulted in a non-significant increase of *in vitro* maturation rate of buffalo oocytes compared to other hormonal additives to the same media. Furthermore, supplementation of maturation media with eCG resulted in a non-significant higher *in vitro* maturation rate of buffalo oocytes compared to FSH and E2 (80.4% for eCG supplemented media vs. 74.0% and 73.0% for FSH and E2, respectively). There was a non-significant difference in the cleavage rate of buffalo oocytes matured in TCM-199 supplemented with either sera or hormones and fertilized either in BO or TALP medium. However, the highest non-significant cleavage rate was achieved when oocytes were matured in TCM-199 supplemented with eCG and fertilized in TALP medium (50%). The overall cleavage rate was not significantly greater in TALP than in BO medium (33.7 vs. 15.5%). It could be concluded that supplementation of maturation media with FCS and/or eCG could successfully improve IVM rate of buffalo oocytes. Furthermore, high cleavage rate could be achieved when oocytes were matured in TCM-199 supplemented with FCS and eCG and fertilized in TALP medium.

Keywords: buffalo, cleavage, IVF, IVM, oocytes.

Introduction

Buffaloes represent an integral part of the agricultural economy in Egypt and some other developing countries. However, buffaloes have low reproductive potential which could be related to the low total number of follicles in the ovary; poor superovulatory response and high percentage of atretic follicles (Haldar and Prakash, 2007; Hufana-Duran *et al.*, 2007).

Efforts have been initiated in recent years to augment the reproductive potential of these animals using biotechnology (Madan *et al.*, 1994). There are new techniques for improving the reproductive efficiency and enhancing the production of genetically superior animals. *In vitro* fertilization (IVF) technology provides an opportunity to produce embryos for genetic manipulation and embryo transfer (Nandi *et al.*, 2002). Producing embryos by IVF can be done based on three subsequent techniques: *in vitro* maturation (IVM) of oocytes, IVF of *in vitro* matured oocytes and then *in vitro* culture (IVC) of fertilized oocytes for cleavage up to blastocyst stage (Palta and Chauhan, 1998; Goswami *et al.*, 2004).

Oocyte maturation is the first and most critical step towards successful *in vitro* embryo production. The culture medium and selection of protein supplements and hormones for IVM play an important role in the subsequent maturation rate, and embryonic development following IVF (Bavister *et al.*, 1992). Several factors such as addition of FSH, LH and their combination to culture media had been considered for maximizing success (Saeki *et al.* 1991).

Little information is available on *in vitro* maturation and fertilization of buffalo oocytes. Preliminary results obtained by several workers (Bacci *et al.*, 1991; Totey *et al.*, 1992) were relatively poor when compared to cattle.

Therefore, the present work aimed to improve *in vitro* maturation rate of buffalo oocytes using different media with addition of protein source (FCS, BSA) or hormones like gonadtropins (FSH, eCG) or estradiol (E2) and to improve *in vitro* cleavage rate using different fertilization media.

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Materials and Methods

This study was carried out at the Dept. of Theriogenology, Faculty of Veterinary Medicine, Mansoura University and Department of Animal Reproduction, Veterinary Division, National Research Center during the period from September 2006 to November 2007.

Collection of oocytes

Ovaries from apparently normal reproductive organs of adult Egyptian buffaloes of unknown breeding history slaughtered in El-Monieb and El-Warak abattoirs were collected within 10 min after slaughter according to Lee and Fukui (1995). Ovaries were washed in fresh, sterile physiological saline to remove any contaminants and rinsed in 70% ethyl alcohol for a few minutes to eliminate surface organisms. After the ovaries had been washed and dried, oocytes were aspirated from 2-8 mm visible follicles using an 18-gauge needle attached to a 10-ml disposable syringe containing 1 ml of pre-warmed modified phosphate-buffered saline (m-PBS) with 5% (v/v) heat inactivated fetal calf serum (FCS). The recovered compact oocyte complexes (COCs) were examined and classified according to their quality (Leibfried and First, 1979) into:
Grade 1: Good oocytes had a homogenous evenly granular ooplasm and were surrounded by compact and dense cumulus cell layers.
Grade 2: Fair oocytes were surrounded by 1-3 layers of cumulus cells.
Grade 3: Denuded oocytes had uneven ooplasm and were completely devoid of cumulus cells around them.

Maturation of oocytes

The good quality buffalo oocytes were used and placed in a four-well culture plate (Nunclon, Denmark) containing a 50 µl droplet of maturation medium, covered with sterilized mineral oil (Sigma, St. Louis, MO, USA). Oocytes were divided into two experiments:

Experiment 1

The oocytes were divided into four groups according to the type of maturation medium supplemented with either 10% FCS or 0.3% BSA (bovine serum albumin) as the following:
Group 1: oocytes (n = 908) matured using Tissue Culture Medium-199 (TCM-199, Hycolne, Logan, Austria) supplemented with either 10% FCS or 0.3% BSA.
Group 2: oocytes (n = 184) matured using Ham's F-10 medium (Sigma) supplemented with either 10% FCS or 0.3% BSA.
Group 3: oocytes (n = 201) matured using Minimum Essential Medium (MEM, Sigma) supplemented with either 10% FCS or 0.3% BSA.
Group 4: oocytes (n = 216) matured using Ferticult medium (FertiPro N.V, Belgium) supplemented with either 10% FCS or 0.3% BSA.

Experiment 2

Based on the results of the first experiment, the second experiment was carried out to investigate the effect of different hormones added to four different media supplemented with 10% FCS as follows:
Group 1: the medium was supplemented with 50 µg/ml equine chorionic gonadotropin (eCG).
Group 2: the medium was supplemented with 50 µg/ml follicle stimulating hormone (FSH).
Group 3: the medium was supplemented with 1 mg/ml estradiol 17β (E2).

All media used were supplemented with 50 µg/ml gentamycin. The culture dishes were placed in a CO_2 incubator (95% relative humidity, 5% CO_2 at 38.5ºC) for 24 h. Maturation was assessed according to nuclear maturation (Gordon, 1995) and determined by the degree of cumulus cells expansion to excellent, good and poor as described by Hunter and Moor (1987).

In vitro cleavage

Frozen thawed semen from a single fertile bull was used. Spermatozoa were capacitated using either Brackett and Oliphant (BO) medium according to Brackett and Oliphant (1975) or TALP medium using swim up technique according to Parrish *et al.* (1986). Each medium was supplemented with 20 µg/ml heparin plus 3.383 mg/ml caffeine as capacitating agents (Niwa and Ohgoda, 1988).

After the matured oocytes had been denuded, they were co-cultured with capacitated spermatozoa (reaching a final concentration of approximately 1-2 x 0^6 spermatozoa/ml) in either BO (Irritani and Niwa, 1977) or TALP medium (Parrish *et al.*, 1986). They were incubated at 38.5ºC under 5% CO_2 in humidified air for 22 h. Fertilization was assessed according to Aoyagi *et al*. (1990). Thereafter, oocytes were transferred to *in vitro* culture droplets (either BO or TALP medium) for further development inside CO_2 incubator (Aoyagi *et al.*, 1990) and cleavage (2-8 cell stage) was checked approximately every 24 h for up to 5 days in order to confirm the occurrence of fertilization. The cleavage rate was recorded according to Iritani *et al.* (1984) as the total number of cleaved oocytes/total number of matured cultured oocytes multiplied by 100.

Statistical analysis

Chi-square test was used to compare maturation and fertilization rates among different groups (Schefler 1979). A 5% significance level was used.

Results

Addition of FCS to all media led to a higher maturation rate, without any significant difference, than BSA did (75.6 vs. 71.3%). However, significantly ($P < 0.5$) higher maturation rate was observed when BSA was added to the FertiCult medium than to other media (Table 1).

Table 1. Effect of supplementation of the maturation medium with serum on *in vitro* maturation rate of buffalo oocytes. Experiment 1.

Supplements	TCM-199	Ham's F-10	MEM	FertiCult	Total
FCS 10%	75.0%^{aA} (318/424)*	78.4%^{aA} (69/88)	71.1%^{aA} (32/45)	85.0%^{aA} (17/20)	75. 6% (436/577)
BSA 0.3%	68.0%^{aA} (85/125)	66.7%^{aA} (26/39)	69.2%^{aA} (18/26)	80.0%^{bA} (60/75)	71.3% (189/265)

*Values within parenthesis indicate no. of matured oocytes/no. of collected ones.
Different small letters within row or capital letters within column denote significant variation ($x^2 = 7.42$; $P < 0.05$), and the same letters within the same row or column denote non-significant variation.

As addition of FCS to different media led to higher maturation rates, in the following experiment we used FCS as a protein source and investigated the addition of different hormones. Although no influence on the maturation rate was observed, addition of eCG to TCM-199, Ham's F-10, MEM or FertiCult media resulted in a non-significant increase of *in vitro* maturation rate of buffalo oocytes compared to other hormonal additives to the same media (Table 2). Furthermore, supplementation of maturation media with eCG resulted in a higher *in vitro* maturation rate of buffalo oocytes than FSH and E2 (80.4% for eCG supplemented media vs. 74.0% and 73.0% for FSH and E2 supplemented media, respectively)**.**

Table 2. Effect of supplementation of the maturation medium with hormone on *in vitro* maturation rate of buffalo oocytes. Experiment 2.

Supplements	TCM-199	Ham's F-10	MEM	FertiCult	Total
eCG	80.4%^{a} (45/56)*	81.8%^{a} (9/11)	83.1%^{a} (64/77)	76.4%^{a} (42/55)	80.4% (160/199)
FSH	73.8%^{a} (194/263)	68.8%^{a} (11/16)	75.8%^{a} (25/33)	76.7%^{a} (23/30)	74.0% (253/342)
E2	67.5%^{a} (27/40)	76.7%^{a} (23/30)	75.0%^{a} (15/20)	75.0%^{a} (27/36)	73.0% (92/126)

*Values within parenthesis indicate no. of matured oocytes/no. of collected ones.
The same letters within the same row and column denote non-significant variation.

There was a non-significant difference in the cleavage rate of buffalo oocytes matured in TCM-199 supplemented with either sera or hormones and fertilized either in BO or TALP medium. However, the highest non-significant cleavage rate was achieved when oocytes were matured in TCM-199 supplemented with eCG and fertilized in TALP medium (5/10, 50%). It appears that the overall cleavage rate was non-significantly greater in TALP than in BO medium (Table 3).

Table 3. *In vitro* cleavage rates of buffalo oocytes previously matured in TCM-199 supplemented with serum or 10% FCS and a hormonal additive.

Fertilization medium	FCS	BSA	eCG	FSH	E2	Total
BO	12.5%^{a} (5/40)*	14.3%^{a} (5/35)	20.0%^{a} (2/10)	14.0%^{a} (7/50)	25.0%^{a} (5/20)	24/155 (15.5%)
TALP	42.1%^{a} (8/19)	25.0%^{a} (5/20)	50.0%^{a} (5/10)	28.6%^{a} (8/28)	33.3%^{a} (2/6)	28/83 (33.7%)
Total	22.03% (13/59)	18.18% (10/55)	35.0% (7/20)	19.2% (15/78)	26.92% (7/26)	

*Values within parenthesis indicate no. of fertilized oocytes/ no. of matured ones.
Values carrying the same superscript letters within the same column or row denote a non-significant variation.

Discussion

Many factors affect *in vitro* maturation of buffalo oocytes. These factors are either the selection of proper maturation medium, the quality of oocytes or the hormones added (De Wit *et al.*, 2000).

The present study revealed that supplementation of maturation media with FCS led to higher maturation rates (75.6% on average), without any significant variation between different media, than BSA (71.3% on average). However, supplementation of FertiCult medium with BSA resulted in a significant increase in the IVM rate of buffalo oocytes (80%) than its addition to other media. These results coincide with those of Chohan and Hunter (2003) and Kumar and Purohit (2004) who observed no difference between serum free group and serum supplemented group for IVM. Moreover, these results are in agreement with those obtained by Totey *et al.* (1993), Holm *et al.* (1999), Curnow *et al.* (2002) and Hakan *et al.* (2004) who recorded beneficial effect of serum supplementation on *in vitro* maturation rate of buffalo oocytes. This might be attributed to the action of serum that promotes cumulus cells-oocyte uncoupling by retaining the hyaluronic acid within the COCs in a manner that results in cumulus mucification. This uncoupling could be responsible for stopping the transfer of oocyte maturation inhibition factor via gap junction (Eppig, 1980).

Supplementation of maturation media with eCG resulted in a non-significant increase of *in vitro* maturation rate of buffalo oocytes in comparison to other hormonal supplements to the same media (80% for eCG supplemented media vs. 74 and 73% for FSH and E2 supplemented media, respectively). These results are comparable to those obtained by Totey *et al.* (1992), Hammam *et al.* (1997) and Kumar and Purohit (2004) who reported a non-significant higher incidence of cumulus expansion when maturation media were supplemented with eCG. Additionally, these results are in agreement with those of Gupta *et al.* (2001) who obtained the highest IVM rate on using TCM-199 plus 10% steer serum supplemented with 40 or 50 i.u. /ml of eCG (83 and 80%, respectively). Furthermore, the present results are in agreement with those obtained by Silvestre *et al.* (2007) who observed non-significant differences in nuclear maturation between groups of COCs matured in medium supplemented with 0.1 i.u./ml FSH and LH when compared with COCs cultured and supplemented with 10 i.u./ml eCG and hCG (68 vs. 71 %, respectively).

The results of this study revealed a non-significant difference in the cleavage rate of buffalo oocytes matured in TCM-199 supplemented with either sera or hormones and fertilized either in BO or TALP medium. However, the highest non-significant cleavage rate was achieved when oocytes were matured in TCM-199 enriched with FCS supplemented with eCG and fertilized in TALP medium (5/10, 50%). The overall cleavage rate was not significantly greater in TALP than in BO medium (33.7 vs. 15.5%).

These results agreed with those reported by Wang *et al.* (1997) and Holm *et al.* (1999) who showed that supplementation of IVM media with FCS or BSA had been found to be beneficial for achieving high *in vitro* fertilization and subsequent development rates. Comparable results were obtained by Totey *et al.* (1993) who showed that supplementation of media with FCS significantly improved the fertilization rate of buffalo oocytes. Conversely, Totey *et al.* (1992), Chauhan *et al.* (1997) and Chohan and Hunter (2003) showed that hormone and/or serum supplementation of TCM-199 did not improve the fertilization rate.

The increased cleavage rate might be attributed to estradiol concentration, which was found to be higher in FCS (Totey *et al.*, 1992, 1993). Moreover, the advantage of FCS might be due to its content of undefined growth promoting components such as Fetuin which are absent in the serum of adult animal and play an important role in preventing the zona hardening during oocyte maturation (Landim-Alvarenga *et al.*, 2002).

These results agreed with those of Totey *et al.* (1992, 1993) and Palta and Chauhan (1998) who showed that mainly FSH is essential for cumulus cell expansion and maturation of buffalo oocytes *in vitro* as FSH enhances the expansion of cumulus cells surrounding the oocytes which in turn enhances sperm capacitation and the fertilization process. Moreover, Saeki *et al.* (1991) showed that total and normal fertilization rates were higher within medium supplemented with the hormones LH (5.0 i.u./ml), FSH (0.5 µg/ml) and E2 (1.0 µm/ml) when compared with control medium (64 vs. 48%). Also, Ali and Sirard (2002) concluded that supplementation of FSH and E2 during IVM of bovine oocytes increased morulae and blastocysts yield following *in vitro* fertilization and *in vitro* culture in defined medium.

It could be concluded that supplementation of maturation media with FCS and eCG could successfully improve IVM rate of buffalo oocytes. Additionally, high cleavage rate could be achieved when oocytes were matured in TCM-199 supplemented with FCS and eCG and fertilized in TALP medium.

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