DEVELOPMENTAL COMPETENCE OF BUFFALO (*BUBALUS BUBALIS*) OOCYTES: EFFECT OF OOCYTES QUALITY, PROTEIN ADDITIVES, HORMONAL SUPPLEMENT AND TYPE OF CAPACITATING AGENTS

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ABSTRACT

Four experiments were conducted to study the effect of media supplements on *in-vitro* maturation, cleavage and embryo development of buffalo oocytes. In experiment 1, oocytes were cultured in TCM-199+10% fetal calf serum (FCS) and kept at 39°C under 5% CO₂ for in-vitro embryo development. In experiment 2, excellent quality oocytes were subjected to TCM-199 enriched with either 10% FCS or estrous buffalo serum (EBS; 20 to 40 pg/ml) and then fertilized using frozen semen in TALP medium containing heparin (0.02 mg/ml) and caffeine (3.89 mg/ml). In experiment 3, oocytes were cultured *in-vitro* maturation (IVM) medium supplemented or not with 20 IU/ml equine chorionic gonadotropins (eCG). Experiment 4 was carried out to examine the suitable capacitating agent, either heparin or caffeine or both. Excellent and good quality oocytes produced higher (P<0.05) maturation and morula development rates. In-vitro maturation and cleavage rates were significantly higher (P<0.05) in IVM medium plus EBS or eCG. Heparin and caffeine produced significantly (P<0.05) higher embryo developmental rates. In conclusion, excellent quality oocytes cultured with either EBS or eCG and fertilized with buffalo spermatozoa capacitated with heparin and caffeine progressively enhanced developmental competence of buffalo oocytes.

Keywords: buffalo, oocytes, eCG, heparin, caffeine

INTRODUCTION

The application of superovulation and embryo transfer in buffaloes has been slow (Mehmood et al., 2011; Kandil et al., 2012). Invitro production of buffalo embryos has been gaining attention for its research and commercial applications (Mehmood et al., 2011). In-vitro embryo production (IVEP) would be an effective technique to improve the efficacy of transferable embryos (Di Francesco, 2010). Aspiration and slicing methods were mostly used for the recovery of buffalo oocytes from abattoir ovaries (Di Francesco, 2010). The efficacy of this method was compared on the basis of cumulus oocyte complexes per ovary (COCs/ovary; Cremonesi et al., 2010). However, these techniques are severely hampered by poor recovery of total oocytes and invitro maturation and fertilization (IVMF; Masudul Hoque et al., 2011). Conditions during in- vitro maturation, fertilization and embryo culture

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(IVMFC) are believed to play a pivotal role in the acquisition of development competence of embryos (Alvarez *et al.*, 2013). Therefore, the present study aimed to increase the developmental competence of buffalo's oocytes by studying the effect of oocytes quality, protein additives, hormonal supplement and type of capacitating agents.

MATERIALS AND METHODS

All chemicals used in the study were reagent grade (Sigma-Aldrich, American Samoa, USA) or as otherwise indicated.

Collection and culture of oocytes

Ovaries were collected at a local abattoir within 20 to 30 minutes after slaughter of buffaloes and transported in a warm saline solution (0.9% NaCl) to a well-equipped laboratory within 2 h. At the laboratory, ovaries were washed 3 times in normal saline containing 100 IU/mL penicillin and 100 ug/mL streptomycin. Non atretic antral follicles (2 to 6 mm diameter) were aspirated with an 18- gauge needle connected to a 10 mL disposable syringe. The aspiration medium consisted of modified phosphate buffer (M-PBS) enriched with sodium pyruvate (0.036 g/mL), 10% fetal calf serum (FCS) and the above mentioned antibiotics. Follicular oocytes were recovered and counted under stereomicroscope. The recovered oocytes were washed 3 times in IVM medium. According to the number of cumulus cell layers and ooplasm morphology, oocytes were divided into three groups as adapted after Dadashpour Davachi et al. (2012): (1) Excellent COCs; (2) Good POCs (partial oocytes complexes); (3) Fair DO (denuded oocytes).

Experimental design

Experimental 1: Effect of oocytes quality on IVMFC of buffalo oocytes. Oocytes (n=320) were cultured in TCM-199 plus 10% FCS and 50 ug/mL gentamicin and covered with mineral oil in CO₂ incubator containing 5% CO₂ and 95% relative humidity at 39°C. Maturation rate was assessed either by the degree of cumulus mass expansion (Srinivasa Prasad *et al.*, 2013) or by staining with 1% aceto-orcein stain (1% orcein in 45% glacial acetic acid) for observation of the 1st polar body (Prentice-Biensch *et al.*, 2012).

Sperm capacitation and IVF

One 0.5 mL straw of frozen buffalo semen was thawed in a water bath at 37°C for 30 seconds. Spermatozoa were washed twice by centrifugation in TALP medium supplemented with 3.89 mg/mL sodium caffeine benzoate and 0.02 mg/mL heparin. Sperm pellet was suspended in 2mL TALP medium enriched with 20 mg/mL bovine serum albumin (BSA) plus the above mentioned additives. The sperm cell concentration was adjusted to 2×10⁶/mL sperm cells (Di Francesco, 2010). A 100 ul aliquot of the sperm cell suspension was placed into a four well cultured dish and covered with warm mineral oil. After maturation, oocytes were washed in the same sperm suspension medium and then 15 to 20 oocytes were transferred into the sperm suspension droplet and cultured under the previous conditions in CO, incubator for 5 h.

After fertilization, oocytes were washed 3 times in IVM medium and then cultured for 6 to 7 days in CO_2 incubator. The cleavage rate and the frequency of morula and blastocyst were recorded.

Experimental 2: Effect of protein additives (EBS) on cleavage and embryo developmental rates of buffalo oocytes. Only excellent quality oocytes (n=237) were washed 3 times in IVM medium then

cultured for maturation in either TCM-199+10% FCS that considered as a control group or in TCM-199+10% estrous buffalo serum (EBS; Jamil *et al.*, 2007). IVM medium was supplemented with 50 ug/mL gentamicin. In both groups, fertilization was performed using frozen thawed buffalo semen capacitated in TALP medium containing heparin (0.02mg/ml) and sodium caffeine benzoate (3.89mg/ml). Maturation, cleavage and embryo developmental rates were carried out as in experiment1.

Experimental 3: Effect of gonadotropin (eCG) added to the maturation medium on cleavage and embryo developmental rates of buffalo oocytes. oocytes (n=290) were classified into two groups; Group 1 washed 3 times in TCM-199 enriched with 10% FCS without gonadotropins and served as a control and Group 2 in which IVM medium was supplemented with 20 IU/ml equine chorionic gonadotropins (eCG; Intergonan®, Intervet, Holland). Fertilization, maturation, cleavage and embryo developmental rates were performed as in experiment 1.

Experimental 4: Selection of suitable capacitating agent added to TALP medium. This experiment (n=210 fertilized oocytes) was carried out to examine the suitable capacitating agent added to TALP medium, either heparin (0.02 mg/ml) or caffeine (3.89 mg/ml) or both. IVM/IVFC

was accomplished as previously mentioned in experiment 1.

STATISTICAL ANALYSIS

Experiments were repeated five times. Data were pooled and analyzed by *Chi-square* test using SPSS 22.0 statistical software (2013).

RESULTS

Experiment 1: The effect of oocytes quality on maturation, cleavage and developmental rates of IVF buffalo oocytes is shown in Table 1. Maturation rate and cleavage rates in COCs and POCs groups was significantly (P < 0.05) higher than with Do type (Figures 1 and 2). In addition, the proportion of embryo that developed to the morula and blastocyst stage was significantly (P < 0.05) higher in COCs and POCs groups than in DO type.

Experiment 2: Table 2 denotes that addition of EBS to the culture medium (TCM-199) produces a significantly (P<0.05) higher maturation and cleavage rates than those cultured in the same medium supplemented with FCS. The proportion of embryos in term of morula and blastocyst doesn't

Table 1. Effect of oocytes quality on the maturation, cleavage and Embryo development rates (%).

Oocytes quality	No. of oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo development rates (%)	
				Morula	Blastocyst
COCs	100	70 (70.00) ^a	35 (50.00) ^a	15 (42.85) ^a	10 (28.57) ^a
POCs	100	65 (65.00) ^a	30 (46.15) ^a	10 (33.34) ^a	5 (16.67) ^b
DO	120	40 (33.34) ^b	10 (25.00) ^b	1 (10.00) ^b	0 (00.00) ^c

Percentages with dissimilar superscripts in the same column are significantly different at P<0.05

Table 2. Effect of estrous buffalo serum (EBS) added to the culture medium (TCM-199) on the maturation, cleavage and embryo developmental rates (%).

Culture condition	No. of oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo development rates (%)	
				Morula	Blastocyst
TCM-199+	100	45 (45.00) ^a	15 (33.34) ^a	4 (26.67) ^a	3 (20.00) ^a
10% FCS	100	43 (43.00)*			
TCM-	137	100 (72.99) ^b	50 (50.00) ^b	15 (30.00) ^a	10 (20.00) ^a
199+10% EBS	13/				

Percentages with dissimilar superscripts in the same column are significantly different at P<0.05

Table 3. Influence of commercially available source of gonadotropins (eCG) on in vitro maturation, cleavage and embryo developmental rates (%).

Culture condition	No. of oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo development rates (%)	
				Morula	Blastocyst
TCM-199+	143	84 (58.74) ^a	25 (29.76) ^a	2	1
10% FCS				$(8.00)^a$	$(4.00)^a$
TCM-199+10%	147		50 (45.46) ^b 10 (20.00) ^b		
FCS+20 IU		110 (74.82) ^b		8 (16.00) ^b	
eCG					

Percentages with dissimilar superscripts in the same column are significantly different at P<0.05

Table 4. Influence of heparin and/or sodium caffeine benzoate addition on the cleavage and embryo developmental rates (%) of buffalo oocytes.

Treatment	No. of fertilized	Cleavage rate	Embryo development rates (%	
	oocytes	(%)	Morula	Blastocyst
Heparin (H)	60	20 (33.34) ^a	2 (10.00) ^a	2 (10.00) ^a
Caffeine (C)	70	25 (35.72) ^a	3 (12.00) ^a	2 (8.00) ^a
H+C	80	45 (56.25) ^b	15 (33.34) ^b	10 (22.23) ^b

Percentages with dissimilar superscripts in the same column are significantly different at P<0.05.



Figure 1. Buffalo embryo developed *in-vitro* to 4-cell stage.

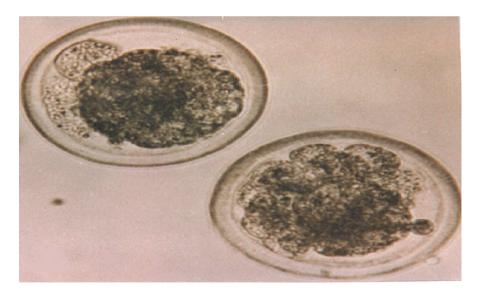


Figure 2. Buffalo embryo developed *in-vitro* to morula stage.

significantly vary between EBS and FCS groups.

Experiment 3: As shown in Table 3, maturation, cleavage and embryo developmental rates up to blastocyst are significantly (P<0.05) higher when eCG added to IVM medium compared with hormonal free medium.

Experiment 4: Table 4 shows that the addition of heparin together with sodium caffeine benzoate to TALP medium resulted in significantly (P<0.05) higher cleavage and embryo developmental rates compared with the use of heparin or caffeine alone.

DISCUSSION

In the present study, data showed that good quality buffalo oocytes surrounded by multilayer of compact investment with a homogenous ooplasm had a significantly higher maturation cleavage, and developmental rates up to blastocyst compared with oocytes of poor quality. This finding identifies the essential role of cumulus cells in promoting normal cytoplasmic maturation of oocytes necessary for fertilization and embryo development of buffalo oocytes. Our results are similar to those previously reported for buffalo oocytes (Gasparrini, 2013). The presence of cumulus cells surrounding the oocyte is essential to facilitate the transport of nutrients and signals into and out of oocytes (Kharche and Birade, 2013). The cumulus cells improve fertilization rate first by providing a capacitatinginducing mechanism and secondly by facilitating the interaction between capacitated spermatozoa and the zona pellucida surface (de Souza et al., 2013). However, Zhang et al. (2012) found that cumulus cells had no influence on fertilization.

The result of this experiment indicated that, addition of EBS to the IVM medium progressively enhanced the developmental competence of buffalo oocytes as compared to FCS additives to the same medium. This is in agreement with the result of Jamil et al. (2007) in buffalo. A possible explanation for the beneficial role of ESB might be a result of its relatively high LH and estradiol levels (Terzano et al., 2012). LH hormone may affect the cytoplasmic maturation of oocytes by increasing the calcium distribution within the ooplasm and promote glycolysis, combined with an increased mitochondrial glucose oxidation metabolism within the oocytes (Silverstre et al., 2007). The beneficial effects of EBS for oocyte maturation may also act via cumulus cells or directly on the oocytes. EBS contains a number of known growth factors that have an important role in the regulation of oocyte maturation, and it also prevents the hardening of the zona pellucida (Jamil et al., 2007). Moreover, Alm et al. (2002) stated that the higher maturation rates of equine oocytes could be due to the increased concentration of insulin like growth factor-1 in estrous mare serum (more than twice that found in FCS).

In the present study, IVM of buffalo oocytes in TCM-199 medium supplemented with eCG increased maturation cleavage, and developmental rates up to blastocyst as compared to control medium. These findings run parallel to those previously reported for buffalo (Hegab *et al.*, 2009). eCG as a source of gonadotropin (more FSH and less LH) stimulation lead to the generation of positive factors that acted on the oocytes to override the inhibitory influence and induced germinal vesicle breakdown (Wang *et al.*, 2009). Therefore, cAMP dependent protein kinase regulated by cumulus cells following FSH- stimulation play a role in the complex mechanism of chromatin

condensation leading to meiotic resumption in bovine oocytes (Tatemoto and Terada, 1998). Moreover, FSH enrichment to the culture medium enhances early embryonic development (Anderiesz *et al.*, 2000).

In this study, the addition of heparin and caffeine to TALP medium resulted in a significantly higher cleavage and embryo developmental rates as compared to the addition of heparin or caffeine alone to the same medium. This is in agreement with the results reported in buffalo by Scholkamy (2002). The role of caffeine may be through increasing the concentration of cAMP which accelerate the rate of capacitation (Breininger *et al.*, 2010); whereas, heparin appeared to be necessary for capacitation and acrosome reaction (Cormier and Bailey, 2003). Heparin stimulates the conversion of proacrosin to acrosin and also it was reported to be responsible for change of calmodulin and calmodulin binding protein at capacitation (Leclerec *et al.*, 1990).

It seems therefore that, there is a synergistic action for both heparin and caffeine in penetration of oocytes *in-vitro*, which depends on their compensatory action to induce capacitation and/or to increase penetration of oocytes (Tajik and Niwa, 1998).

CONCLUSIONS

Excellent quality oocytes cultured in IVM medium supplemented with either protein additives (EBS) or hormonal supplement (eCG) and fertilized with capacitated buffalo spermatozoa in TALP medium enriched with heparin and caffeine progressively enhanced developmental competence of buffalo oocyte.

ACKNOWLEDGEMENT

The study was conducted in the Veterinary Research Division, National Research Centre, Dokky, Egypt.

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