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INFLUENCE OF OOCYTE QUALITY AND DIFFERENT MEDIA SUPPLEMENT ON IN VITRO MATURATION, CLEAVAGE AND EMBRYO DEVELOPMENT OF BUFFALO OOCYTES

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SUMMARY

The present study was designed to examine the influence of oocyte quality and different media supplement on in vitro maturation, cleavage and embryo development of buffalo oocytes. Four experiments were conducted. In experiment 1, oocytes were classified by number of cumulus cell layers and morphology of ooplasm as excellent, good or fair. Oocytes were cultured for in vitro maturation, fertilization and embryo culture (IVMFC) in TCM-199 plus 10%FCS (fetal calf serum). In experiment 2, excellent quality oocytes were cultured for maturation in TCM-199 enriched with either 10% FCS or estrous buffalo serum (EBS) and then fertilized using frozen thawed buffalo semen capacitated in BO medium containing heparin and caffeine. In experiment 3, oocytes were classified into two groups; group (1) was without gonadotropins and serve as a control; group (2) in which IVM medium was supplemented with 20 iu/ml gonadotropins (PMSG). Experiment 4 was carried out to examine the suitable capacitating agent which added to BO medium, either heparin or sodium caffeine benzoate or both. In all experiments, oocytes were kept at 39°C under 5% CO_2 for IVMFC and examined for cleavage and embryo development (morula and blastocyst). Excellent and good quality oocytes produced a higher ($p<0.05$) maturation and cleavage rates than poor quality oocytes. Blastocyst production rate was also higher ($p<0.05$) for excellent as compared with good quality oocytes. In experiment 2, the in vitro maturation and cleavage rates were significantly higher ($p<0.05$) in IVM medium plus 10% EBS than those cultured in 10% FCS. In experiment 3, the addition of PMSG to maturation medium increased ($p<0.05$) developmental competence of buffalo oocytes (IVMFC) compared with control

medium. In experiment 4, the addition of heparin together with caffeine to BO medium produced significantly ($p<0.05$) higher cleavage and embryo developmental rates compared with heparin or caffeine alone. In conclusion, excellent quality oocytes cultured in IVM medium supplemented with either protein additives (EBS) or hormonal supplement (PMSG) and fertilized with capacitated buffalo spermatozoa in BO medium enriched with heparin and caffeine progressively enhanced developmental competence of buffalo oocyte.

INTRODUCTION

The application of superovulation and embryo transfer in buffaloes has a limited success(Madan et al.,1996) .There is, therefore, an increasing interest in the large scale production of buffalo embryo through in vitro maturation, fertilization and culture(IVMFC) of faster multiplication of superior germplasm by embryo transfer through the use of oocytes collected by ovum pick-up. The IVMFC procedures have been successfully used for routine production of embryos from slaughterhouse ovaries in buffaloes (Chauhan et al., 1998). The practical application of these techniques is, however, severely hampered by very poor recovery of total oocytes and IVMF (Madan et al., 1994). Conditions during IVMFC are believed to play a role in the acquisition of developmental competence of embryos (First and Parrish, 1987; Brackett et al., 1989).

Therefore, the present study aimed to increase the developmental competence of buffalo's oocytes by studying the effect of the oocyte quality, type of protein additives (estrous buffalo serum), hormonal supplement (pregnant mare serum) and type of capacitating agents (heparin and/ or sodium caffeine benzoate).

MATERIALS AND METHODS

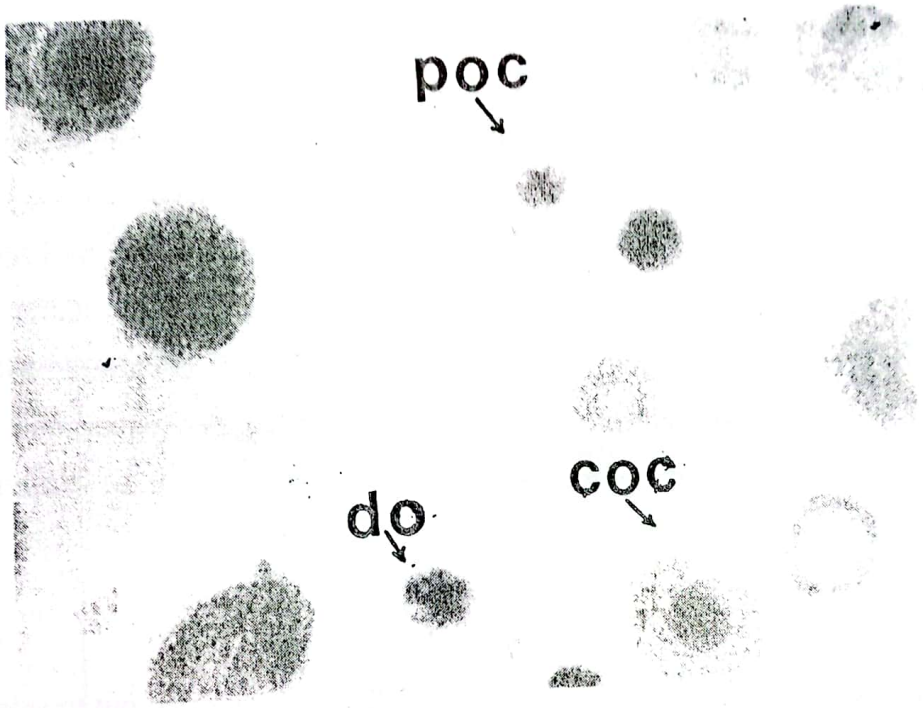
All chemical and media, unless otherwise indicated, were purchased from Sigma Chemical Company (St. Louis, MO, USA). Follicle stimulating hormone (FSH) was obtained from pregnant mare serum (Folligon®, Intervet, and Holland). Estrous buffalo serum (EBS) was prepared as described earlier by Madan et al. (1994).

I. Collection and culture of oocytes

Ovaries were collected at a local abattoir within 20-30 minutes after slaughter of buffalo-cows, and transported in a warm saline solution (0.9% NaCl) within 2-3 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 (2-6 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 (Kim and park, 1990): (1) Excellent COCs (cumulus oocytes complexes); (2) Good POCs (partial oocytes complexes); (3) Fair DO (denuded oocytes) as shown in Figure 1.

II. Experimental design



Fig(1): Quality of recovered immature buffalo oocytes. COCS (cumulus oocytes complexes); (b) POCs (partial oocytes complexes); (c) DO (denuded oocytes).

Experiment 1:

This experiment was designed to study the effect of oocytes quality on IVMFC of buffalo oocytes. According to their groups, oocytes were cultured in TCM-199 plus 10% EBS and 50 ug/ml gentamicin and covered with mineral oil in CO_2 incubator containing 5% CO_2 and 95% relative humidity at 39°C. Maturation rate was assessed either by the degree of cumulus mass expansion (Kobayashi et al.,1994) or by staining with 1% acetoorcine stain for observation of the 1st polar body (Hurtt et al., 2000).

Sperm capacitation and in-vitro fertilization (IVF):

One straw (0.5 ml) of frozen buffalo semen was

thawed in a water bath at 37°C for 30 sec. Spermatozoa were washed twice by centrifugation in BO medium (Brackett and Oliphant, 1975) supplemented with 3.89 mg/ml sodium caffeine benzoate and 0.02 mg/ml heparin. After washing, the sperm pellet was suspended in 2 ml BO medium enriched with 20 mg/ml bovine serum albumin (BSA) plus the above mentioned additives. The sperm cell concentration was adjusted to 5 - 8x10^6 sperm cells /ml (Niawa et al., 1991). 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 15-20 oocytes were transferred into the sperm suspension droplet and cultured under the previous conditions in CO_2 incubator for 5 h.

In vitro culture (IVC):

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

Experiment 2:

This experiment was carried out to evaluate the effect of protein additives (EBS) on cleavage and embryo developmental rates of buffalo oocytes. Only COCs were washed 3 times in IVM medium then cultured for maturation in: (a) TCM-199 + 10% FCS; (b) TCM-199 +10% EBS. IVM medium was supplemented with 50 ug/ml gentamicin. In both group, IVF was performed in BO medium as mentioned in experiment1. For IVC, oocytes in each group were cultured in the same medium for IVM. Maturation, cleavage and embryo developmental rates were carried out also as in experiment1.

Experiment 3:

This experiment was designed to measure the effect of gonadotropin (PMSG) added to the maturation medium on cleavage and embryo developmental rates of buffalo oocytes. COCs were washed 3 times in TCM-199 enriched with (a) 10% FCS served as control without gonadotropins; (b) 10% FCS plus 20 iu/ml PMSG. IVF and IVC were performed as in experiment 1. Maturation, cleavage and embryo developmental rates were reported.

Experiment 4:

This experiment was carried out to select the suitable capacitating agent which added to BO medium, either heparin or sodium caffeine benzoate; or both together. IVM/IVFC was carried as previously mentioned in experiment1. Cleavage rate and embryo developmental rates of buffalo oocytes were recorded.

III. Statistical analysis:

The experiment was repeated for five replications. Data were pooled and analyzed by Chi-square test with a probability level of $P< 0.05$ considered significant according to Snedecor and Cochran (1976).

RESULTS

Experiment 1:

The effect of oocytes quality on maturation, cleavage and developmental rates of IVF buffalo oocytes are shown in Table, 1. Maturation and cleavage rates in COCs and POCs groups were significantly higher ($p<0.05$) than with DO type (Figures, 2 and 3).In addition, the proportion of embryos that developed to the morula and blastocyst stage were higher ($p<0.05$) in COCs and POCs groups than in DO type.

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

Oocytes quality	Number of Oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo developmental 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)^{a}	10 (25.00)^{b}	1 (10.00)^{b}	0 (00.00)^{c}

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

Fig.(2): In vitro matured buffalo oocyte showed expansion of cumulus cells.

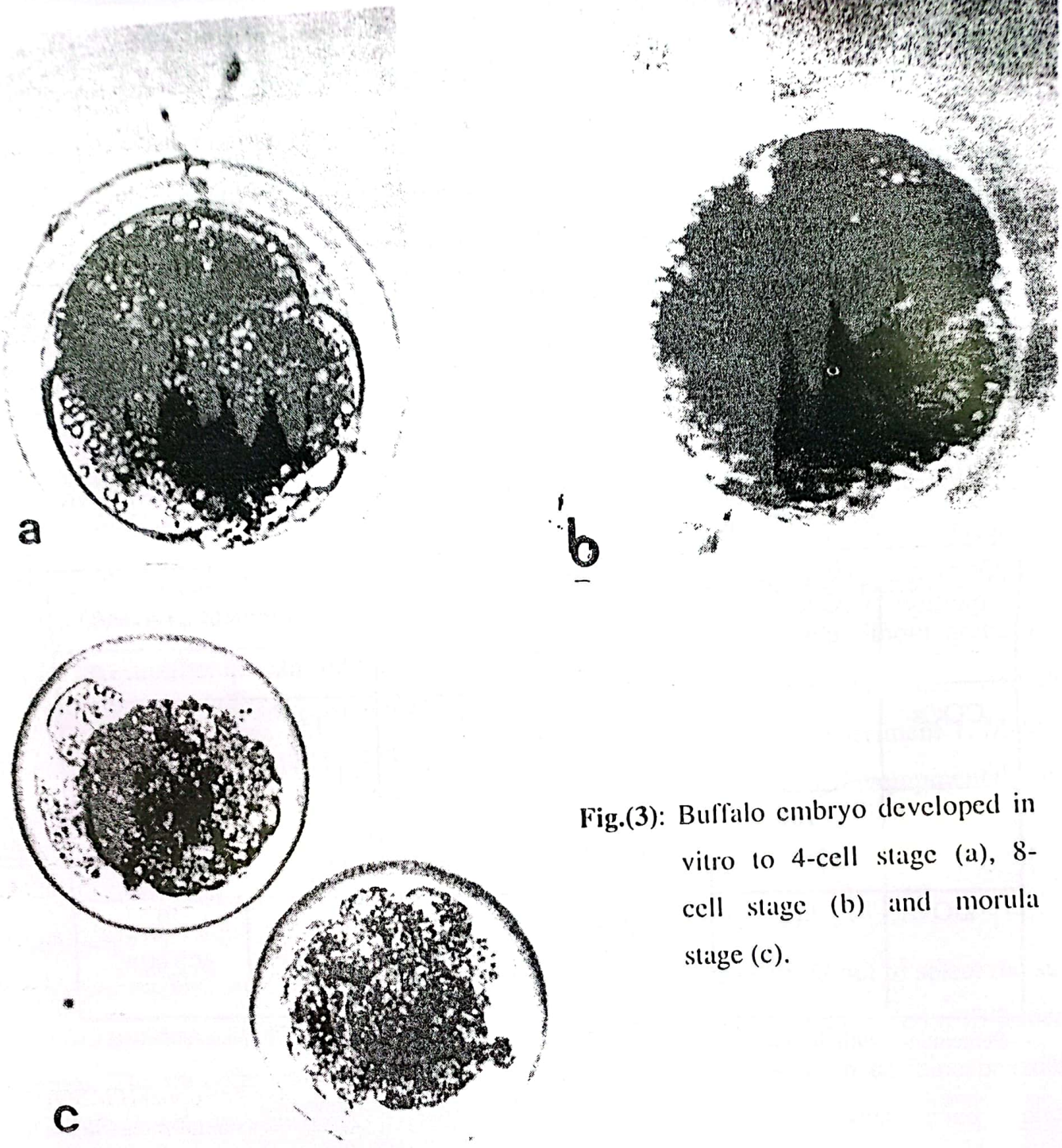


Fig.(3): Buffalo embryo developed in vitro to 4-cell stage (a), 8-cell stage (b) and morula stage (c).

Experiment 2:

Table 2 denoted that addition of EBS to the culture medium (TCM-199) produces a higher ($p<0.05$) 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 significantly vary between EBS and FCS groups.

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	Number of Oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo developmental rates (%)	
				Morula	Blastocyst
TCM-199 +10% FCS	100	45 (45.00)[a]	15 (33.34)[a]	4 (26.67)[a]	3 (20.00)[a]
TCM-199 +10% EBS	137	100 (72.99)[b]	50 (50.00)[b]	15 (30.00)[a]	10 (20.00)[a]

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

Experiment 3:

As shown in table 3, maturation, cleavage and embryo developmental rates up to blastocyst are significantly (p<0.05) higher when PMSG is added to IVM medium compared with hormonal free medium.

Experiment 4:

Table 4 showed that the addition of heparin together with caffeine to BO medium resulted in significantly (p<0.05) higher cleavage and embryo developmental rates compared with the use of heparin or caffeine alone.

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

Culture condition	Number of Oocytes cultured	Maturation rate (%)	Cleavage rate (%)	Embryo developmental rates (%)	
				Morula	Blastocyst
TCM-199 +10% FCS	143	84 (58.74)[a]	25 (29.76)[a]	2 (8.00)[a]	1 (4.00)[a]
TCM-199 +10% FCS+20 iU PMSG	147	110 (74.82)[b]	50 (45.46)[b]	10 (20.00)[b]	8 (16.00)[b]

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	Number of fertilized oocytes	Cleavage rate (%)	Embryo developmental rates (%)	
			Morula	Blastocyst
Heparin (H)	60	20 (33.34)^{b}	2 (10.00)^{b}	2 (10.00)^{b}
Caffeine (C)	70	25 (35.72)^{b}	3 (12.00)^{b}	2 (8.00)^{b}
H + C	80	45 (56.25)^{a}	15 (33.34)^{a}	10 (22.23)^{a}

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

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 (Suzuki et al., 1992; Nandi et al., 1998 and Abdoon et al., 2001). However, Behalova and Greve (1993) and Hawk et al (1992) found that, cumulus cells had no influence on fertilization. The presence of cumulus cells surrounding the oocyte is essential to facilitate the transport of nutrients and signals into and out of oocytes (Moor and Seamark, 1986). The cumulus cells improve fertilization rate first by providing a capacitating- inducing mechanism and secondly by facilitating the interaction between capacitated spermatozoa and the zona pellucida surface (Goud et al., 1998).

The result of our experiment indicated that, addition of EBS to the IVM medium progressively enhanced the developmental competence of buffalo oocytes as compared to FCS additives in the same medium. This is in agreement with the result of Scholkamy (2002) in buffalo. A possible explanation for the beneficial role of EBS might be a result of its relatively high LH and estradiol levels (Sanbuissho and Threlfall, 1990; Schellander et al., 1990); 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 (Brackett and Zuelka, 1993). Moreover, Alm et al. (2002) concluded that the higher maturation rates of equine oocytes could be due to the increased concentration of insulin like growth factor I in estrous mare serum (more than twice that found in FCS).

In the present work, IVM of buffalo oocytes in TCM-199 medium supplemented with PMSG increased maturation, cleavage, and developmental rates up to blastocyst as compared to control medium. These findings run parallel to those previously reported for buffalo (Abdoon et al., 2001; Ravindranatha et al., 2002). PMSG 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 (Downs, 1993). 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 (Eyestone and Boer, 1993).

In our study, the addition of heparin and caffeine to BO 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 Hegab (1991) and Scholkamy (2002). The role of caffeine may be through increasing the concentration of cAMP which accelerate the rate of capacitation (Parrish et al., 1985); whereas, heparin appeared to be necessary for capacitation and acrosome reaction (Fukui et al., 1990). 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 (Leclerc 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 (Niawa and Oghoda 1988; Kim et al., 1990). In conclusion, IVM of excellent quality oocytes (COCs) in medium supplemented with either protein additives (EBS) or hormonal supplement (PMSG) and fertilized with capacitated buffalo spermatozoa in BO medium enriched with heparin and caffeine improved developmental competence of buffalo oocyte.

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