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EFFECT OF ADDITION OF FOLLICULAR FLUID, OVIDUCTAL OR GRANULOSE CELL CO-CULTURE ON CLEAVAGE AND EMBRYONIC DEVELOPMENT OF BUFFALO OOCYTES

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SUMMARY

The present study was undertaken with aim to study the role of addition of follicular fluid, oviductal cell and granulose co-culture on cleavage and embryonic development of buffalo oocytes. Cumulus oocyte complexes (COCs) were obtained from slaughterhouse ovaries by aspiration method. Oocytes were cultured for in vitro maturation in TCM-199 plus 10%fetal calf serum (FCS) for 24h in CO_2 incubator. Oocytes were fertilized with frozen-thawed semen capacitated in Brackett and Oliphant (BO) medium contained heparin and caffeine. After 5h of co-incubation, sperm attached matured oocyte were transferred to maturation media drops for further development and co-culture according to experimental design, Group 1 without co-culture (control), group 2 (Bovine oviductal epithelial cells, BOECs) and group3 (Granulose cells GCs) . In another group of experiments, matured oocytes were fertilized with frozen-thawed semen capacitated in BO medium contained 20% Buffalo follicular fluid (BFF) then transferred to maturation media drops for further development. Oocytes were observed for cleavage after 48h of fertilization. The cleavage rate and the frequency of morula and blastocyst were recorded. Results revealed that, addition of BOECs to the culture media produced significantly ($p<0.05$) higher cleavage and embryonic developmental rates (Morula and blastocyst) compared to control group (35.0, 15.0 vs 10.0, 10.0%, respectively). Morula and blastocyst production rates were significantly higher in TCM-199+10% FCS+GCs than those obtained in TCM-199+10%FCS (33.34, 13.34 vs 10.00, 0.0%; $p<0.05$, respectively). However the cleavage rates were similar in

both groups. The fertilization of oocytes with semen capacitated in BO medium contained 20% BFF were resulted in significantly ($p<0.05$) higher embryo developmental rates (Morula and blastocyst) compared with the use of heparin and caffeine in the same medium (22.23, 10.0 vs. 10.0, 0.0%, respectively). In conclusion, oviductal or granulose cell co-culture had a marked effect on cleavage and embryonic development of buffalo oocytes. Also, addition of buffalo follicular fluid to BO medium could increase the percentage of morula and blastocyst.

INTRODUCTION

Recent advances in vitro maturation and fertilization of oocytes among farm animals have generated the scope of better exploitation of their existing reproductive potentials. The emerging interest of workers to generate embryos in vitro and subsequent transfer in foster mothers has lead to the development of several media and various additives to optimize embryo generation. However, the date knowledge about the exact requirement of growing embryos through its different developmental stages is not complete (Yadav et al., 1998).Efforts have been made to supplement the culture media with natural additives like follicular fluid (Tajik et al., 2008) and oviductal cells of other species(Madan et al., 1994). Recent approach using various somatic cell embryo co culture systems have circumvented the in vitro block to development and have permitted bovine zygotes to be cultured to morula and blastocyst stages (Rexroad, 1989). The two somatic cell types often used for bovine embryo co-culture are cumulus/ granulose cells (Xu et al., 1992) and oviduct epithelial cells (Madan et al., 1994). Therefore, the present study aimed to investigate the influence of follicular fluid, oviductal and granulose cell co-culture on developmental capacity of buffalo oocytes.

Key words: buffalo oocyte, granulose, oviductal cell, IVM, IVF.

MATERIALS AND METHODS

2.1. Preparation of buffalo oviductal epithelial cells (BOECs)

Oviducts of buffaloes were collected from el-moneib abattoir. The oviducts of the animals with corpus hemorrhagicum on their ovary were only selected. They were transported to the laboratory in thermos containing normal saline plus antibiotic (100ug/ml streptomycin and 100 iu/ml penicillin) at 25°C. The oviducts were dissected free from adjacent connective tissue and washed with normal saline. They were held by artery forceps and its contents were squeezed out by a pair of forceps into a centrifuge tube containing 10 ml of TCM-199 plus 10%

FCS+50ug/ml gentamicin (maturation media). The content was mixed by gently inverting the tube several times for 10 min, allow to sediment and the supernatant was discarded. After repeating the procedure 5 times the pellet was suspended in TCM-199 with abovementioned additives. The cells were cultured for 24h in 100ul drops of maturation media containing 100-200 oviductal cells covered under mineral oil, before putting them with mature inseminated oocytes (Hamam, 1997).

2.2. Preparation of granulose cells (GCs)

Granulose cells were prepared from the sediment at the time of oocyte collection. The cells were washed 2 to 3 times in maturation media, before adding them with mature inseminated oocytes.

2.3. Preparation of buffalo follicular fluid (BFF):

The follicular contents of large follicles (5-7mm in diameter) were aspirated using disposable syringe, left in graduated cylinder to settled, and were aspirated the supernatant. The supernatant was heat-inactivated at 56 °C for 30 min, centrifuge at 3000 rpm for 5 min (Tajik et al., 2008). The supernatant of BFF was diluted 1:5 (v/v) with BO medium and used for incubation of semen for 1 hr in Co_2 incubator before IVF of oocytes. Preliminary experiments were carried out to evaluate the addition of BFF (20%) to BO medium compared to BO medium enriched with heparin plus caffeine on cleavage and embryo developmental rates.

2.4. Oocyte maturation (IVM)

Buffalo ovaries (n=100) were collected from el-moneib slaughter house in normal saline contained 100ug/ml streptomycin and 100 iu/ml penicillin at 37c kept in thermos. The ovaries were brought to the laboratory and washed twice with normal saline. The oocytes were recovered by aspiration of the visible follicles with 18-gauge needle fitted on disposable syringe filled with 1ml of aspiration media (modified phosphate buffer saline, M-PBS, pH, 7.2). M-PBS was supplemented with 3% fetal calf serum (FCS, Sigma, USA) with above mentioned antibiotics. Follicular oocytes were recovered and counted under stereomicroscope. According to the number of cumulus cell layers and ooplasm morphology, oocytes were divided into three groups (Kim and Park, 1990). (1) COCs (completely invested with cumulus cells) were selected; (2) Good POCS (partial oocytes complexes);(3) Fair DO (Denuded oocytes) . Only COCs were washed 3 times using maturation media (TCM-199, with Earle's salts, L-glutamine and 25 mM HEPES, Sigma, USA) enriched with 10% FCS plus 50ug/ml gentamicin and then cultured for maturation. Ten to fifteen oocytes were cultured in 100 µl of maturation media drops covered under

mineral oil (sigma) and incubated in 5% CO_2 at 38.5°c for 24 hours. Maturation of oocytes was assessed by appearance of first polar body and expansion of cumulus cell (Kobayashi et al., 1994).

2.5. Sperm capacitation and in vitro fertilization (IVF)

One straw 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 (Brakett and Oliphant, 1979) supplemented with 3.89mg/ml sodium caffeine benzoate and 0.02 mg/ml heparin. After washing, the Spermatozoa were suspended in 2ml 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 µl aliquot of the sperm cell suspension was placed into four well cultured dishes and covered with warm mineral oil. After maturation, oocytes were washed in the BO medium for fertilization. After 5h of co-incubation, sperm attached matured oocyte were transferred to maturation media drops for further development and co-culture with the according to following experimental design, Group 1 without co-culture (control), group 2 (BOECs) and group3 (GCs). The media were changed every 48h and then cultured for 6-7days in CO_2 incubator. Oocytes were observed for cleavage after 48h of fertilization. The cleavage rate and the frequency of morula and blastocyst were recorded.

2.6. Statistical analysis

The experiment was replicated 4 times. The data were analyzed using Chi-square analysis according to Snedecor and Cochran (1976).

3. RESULT

The results of the effect the oviductal cell co-culture are shown in Table 1. The cleavage rate was found only in 20.0% of matured oocytes in TCM-199+10% FCS, while 40.0% of matured oocytes in TCM-199+10%FCS+BOECs showed cleavage with significant difference ($p<0.05$). A higher significantly ($p<0.05$) percentage of morula and blastocyst formation was obtained when culture medium (TCM-199+10%FCS) supplemented with BOECs compared to those cultured in TCM-199+10%FCS alone (35.0, 15.0 vs 10.0, 10.0%, respectively).

Table 1. Effect of addition of buffalo oviduct epithelial cells (BOECs) co-culture on cleavage and embryo developmental rates (%).

Culture condition	Total no. of inseminated oocytes	Cleavage rate (%)	Embryo developmental rates (%)	
			Morula	blastocyst
TCM-199+10% FCS	50	10(20.0)[a]	1(10.0)[a]	1(10.0)[a]
TCM-199+10% FCS+BOECs	50	20(40.0)[b]	7(35.0)[b]	3(15.0)[b]

Within the same column values with different superscripts (a, b) are significantly different at least ($p<0.05$).

Table 2 denoted that, addition of granulose cells (GCs) to culture medium (TCM-199+10%FCS) produced a higher cleavage rates (30.0%) than those cultured in the medium alone (20.0%) but without significant difference. The proportion of embryo in term of morula and blastocyst was significantly ($p<0.05$) vary between TCM-199 plus 10% FCS+GCs and TCM-199+10%FCS (33.34, 13.34 vs 10.0, 0.0%, respectively).

Table 2. Effect of addition of granulose cells (GCs) co-culture on cleavage and embryo developmental rates (%).

Culture condition	Total no. of inseminated oocytes	Cleavage rate (%)	Embryo developmental rates (%)	
			Morula	blastocyst
TCM-199+10% FCS	50	10(20.0)[a]	1(10.00)[a]	0(0.00)[a]
TCM-199+10% FCS+GCs	50	15(30.0)[a]	5(33.34)[b]	2(13.34)[b]

Within the same column values with different superscripts (a, b) are significantly different at least ($p<0.05$).

Supplementation of BFF (20%) to BO medium resulted in cleavage rates similar to those obtained in BO+heparin+Caffeine (20.0 and18.0%, respectively). However, a significantly ($p<0.05$) higher embryo developmental rates (Morula and blastocyst) were obtained from BO+ BFF (20%) compared with the use of heparin and caffeine in the medium (22.23, 10.0 vs 10.0, 0.0%, respectively) as shown in Table 3.

Table 3. Effect of addition of buffalo follicular fluid (BFF) co-culture on cleavage and embryo developmental rates (%).

Culture condition	Total no. of inseminated oocytes	Cleavage rate (%)	Embryo developmental rates (%)	
			Morula	blastocyst
BO+heparin+Caffeine	50	10(20.0)[a]	1(10.00)[a]	0(0.00)[a]
BO+ BFF (20%).	50	9(18.0)[a]	2(22.23)[b]	1(13.34)[b]

Within the same column values with different superscripts (a, b) are significantly different at least ($p<0.05$).

DISCUSSION

The results of the present study clearly indicated that addition of granulose and oviduct epithelial cells to the culture medium could maintain the development of oocytes to the blastocyst stage in vitro. The highest percentage of cleavage, morula and blastocyst was obtained when culture medium (TCM-199) supplemented with BOECs or GCs. Similar finding was obtained by Hamam et al. (1997) in cattle. Furthermore, Yadav et al. (1998) concluded that, buffalo oviductal cells co culture had a marked effect on cleavage and development of goat IVF. Xiaoxia et al.,(1991) indicated that oviductal cells were more effective than cumulus in promoting bovine embryo development , while others have reported cumulus that, and oviductal cells were equally effective (Rodriguez et al., 1991). This effect may be because; the oviductal cells secrete specific proteins that are only produced during the 4 or 5 day after estrus (Gandolfi et al., 1989). These proteins cross the zona pellucida and associate with individual blastomeres, suggesting a role in early embryonic development. Mermillod et al. (1993) reported that a low molecular weight (< 10,000) component of serum-

free, oviductal cell conditioned medium promoted bovine embryo cleavage and a higher molecular weight (>10,000) component enhanced blastocyst formation. Working in similar lines, Kitiyanant et al. (1995) observed that buffalo oocytes could effectively be matured and cleaved to the transferable stage using bovine oviductal cells. In the present study addition of granulosa cells at the time of fertilization resulted in significantly proportion of morula and blastocyst than that observed for granulose free culture media. Similar finding was obtained by Fukui (1989); Younis and Brackett (1991) and Hamam (1997). It was observed that cumulus or granulosa are necessary at the time of in vitro insemination to maximize incidence of the acrosome reactions in frozen-thawed bovine spermatozoa and subsequent development in vitro resulting in fertilization (Fukui, 1989). In the absence of cumulus cells, the fertilizing sperm cells penetrated ova less effectively than in the presence of cumulus cells (Younis and Brackett 1991). The later authors recorded that co-culture of bovine zygotes with cumulus cells monolayers enhanced development as compared to culture in medium alone thereby confirming previous work (Goto et al., 1988; Utsumi et al., 1988).Follicular fluid is instrumental in the nutritional and developmental support of the oocyte. Follicular maturation and the maturation of its oocyte are parallel events and also functionally related (Tajik et al., 2008). In the present study regarding development of embryos to morula and blastocyst, a significant improvement was observed when the medium enriched with 20% BFF compared to control one. Recent studies have shown that the follicular fluid derived from small, medium, large and pre-ovulatory follicles supplemented to the maturation medium at the range of 10% (Carolan et al., 1996; Elmileik et al., 1995 and Sirard et al., 1995), 20% (Romero-Arredondo and Seidel, 1996) and 100% (Choi et al., 1998) improved the developmental capacity of bovine oocytes. In swine, also a medium of 100% follicular fluid supplemented with FSH 0.12 IU mL^{-1} was used for oocyte maturation, markedly improved male pronuclear formation (Naito et al., 1988 and 1989). Incubation of spermatozoa for 1hour in 20% BFF before addition of matured oocytes, relatively elevated the cleavage rates (18.0%) similar to those obtained in BO free BFF (20.0%). It is appeared that the effect may be due to some factors contained in the follicular fluids which may be protein in nature (Kato and Iritani, 1993) that may play a role in capacitation processes of spermatozoa. Mattioli et al. (1988) have reported that follicles contained medium could keep gap junctions between oocyte and cumulus cells tight and this caused

improved male pronuclear formation after IVF in the pig. They postulated that the existence of soluble factors secreted by ovarian follicles were responsible for this action. In conclusion, oviductal or granulose cell co-culture had a marked effect on cleavage and embryonic development of buffalo oocytes. Also, the addition of buffalo follicular fluid to BO medium could increase the percentage of morula and blastocyst.

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تأ ثير الخلايا الطلائية لقناة المبيض والخلايا المحببة المحيطة بالبويضات والسائل الحويصلى المضاف الى البيئات الصناعية على الاخصاب والنمو وتكوين الاجنة لبويضات الجاموس

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تم تصميم هذا العمل لدراسة تأ ثير الخلايا الطلائية لقناة المبيض و الخلايا المحببة المحيطة بالبويضات والسائل الحويصلى فى المضافة الى البيئات الصناعية على الاخصاب والنمو وتكوين الاجنة لبويضات الجاموس . تم الحصول على البويضات الجيدة من المبايض الجاموس التى تم تجميعها من السلخانة بطريقة الشفط وتم زراعها فى الحضانة ثانى اوكسيد الكربون لمدة ٢٤ تم . تم تلقيح البويضات معمليا بسائل منوى مسال 10%fetal calf serum (FCS)+TCM-199 تم تحفيزة فى وسط براكت والفين الذى يحتوى على الهيبارين والكافين . بعد ٥ ساعات من التحضين تم نقل البويضات المخصبة وزراعتها فى الميديا السالف ذكرها مع بعض الاضافات على نوعية التجربة . المجموعة كما هى (المجموعة الضابطة). المجموعة الثانية تمت أضافة الخلايا الطلائية لقناة المبيض . المجموعة الثالثة اضيفت اليها الخلايا المحبية المحيطة بالبويضات وفى تجربة اخرى تمت أضافة ٢٠% من السائل الحويصلى الى فى وسط براكت والفين من غير أضافة الهيبارين والكافين لسائل المنوي المسال وتم تلقيح البويضات السابق انضاجها و تمت متابعة البويضات الملقحة لحساب معدل الانقسام وتكوين مرحلتى الموريولا والبلاستوست فى كل المجاميع. أشارت النتائج الى أرتفاع معدل الانقسام وتكوين مرحلتى الموريولا والبلاستوست عند أضافة الخلايا الطلائية لقناة المبيض بالمقارنة بمجموعة الكنترول (٣٥.٠ و١٥.٠ ضد ١٠.٠ و١٠.٠% على التوالى). كما لوحظ زيادة معدل تكوين الموريلا والبلاستوست المتكونة من البويضات الملقحة والمضاف اليها الخلايا المحببة المحيطة بالبويضات بالمقارنة بالكنترول (٣٣.٣٤ و١٣.٣٤ ضد ١٠.٠ و ٠.٠% على التوالى). لوحظ أن معدل تكوين الموريلا والبلاستوست من البويضات الملقحة من السائل المنوى المسال المحفز بأضافة ٢٠% من السائل الحويصلى الى وسط براكت والفين أعلى معنويا بالمقارنة بالبويضات الملقحة الهيبارين والكافين (٢٢.٢٣ و١٠.٠ ضد ١٠.٠ و٠.٠ % على التوالى) . نستنتج من ذلك أضافة الخلايا الطلائية لقناة المبيض و الخلايا المحببة المحيطة بالبويضات الى الاوساط يؤدى الى رفع معدلات الانقسام وتكوين الموريلا والبلاستوست. كما أن أضافة من السائل الحويصلى الى وسط براكت والفين يؤدي الى زيادة تكوين مرحلتى الموريلا والبلاستوست.