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FACTORS AFFECTING IN VITRO PRODUCTION OF BUFFALO EMBRYOS

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SUMMARY

The present study was designed to examine the influence of oocyte quality, culture media and gonadotropins on cleavage rate and development of in vitro fertilized buffalo oocytes. Three experiments were conducted. In Experiment 1; oocytes were classified by number of cumulus cell layers and morphology of the oopbsm as Good, Fair or Poor. Oocytes were cultured for IVM, IVF and IVC in CR1aa medium. In Experiment 2, good quality oocytes were cultured for maturation in: (1) CR1aa; (2) CR2aa; (3) TCM-199; (4) MEM and (5) RPMI-1640, and then fertilized using frozen thawed buffalo spermatozoa in CR1aa. After fertilization, oocytes were cultured in the same medium used for maturation. In Experiment 3, oocytes were classified into 3 groups: Group (1) without gonadotropin and served as control; Group (2) in which IVM medium was supplemented with 10 μg/ml FSH; Group (3) in which IVM medium was supplemented with 10 IU/ml PMSG. In all experiments, oocytes were kept at 38.5°C under 5% CO_2 for IVM, IVF, IVC and examined for cleavage and embryo development rates on day 3 and 8, respectively. Good and fair quality oocytes produced a higher (P<0.01) cleavage rate than poor quality oocytes. Morula production rate was also higher (P<0.01) for good as compared to fair quality oocytes. Embryo development with poor quality oocytes was arrested at the 2 to 16-cell stage. In Experiment 2, the cleavage rate was significantly higher(P<0.05) in CR1aa than CR2aa, and significantly higher (P<0.01) than TCM-199, MEM and RPMI-1640. The numbers of morulae and blastocysts were higher (P<0.01) for oocytes cultured in CR1aa and CR2aa media than TCM-199 or MEM. In Experiment 3, the addition of FSH or PMSG to the maturation medium significantly increased (P<0.01) cleavage and developmental rates of buffalo embryo compared to control media . In conclusion, the IVM of good quality buffalo oocytes in CR1aa or CR2aa medium and the addition of FSH or PMSG in maturation medium produced higher cleavage and developmental rates of IVF buffalo embryos.

Key words: Buffalo, IVF, oocyte quality, culture media, gonadotropins, cleavage and embryo developmental rates.

INTRODUCTION

The in vitro production (IVP) of embryos is desirable in strategies to enhance reproductive and genetic advances especially in species like buffalo. Reports have indicated that classification of bovine oocytes based on visual assessment of the compactness and quality of follicle cells can be used to select immature oocytes capable of maturation, fertilization and cleavage invitro (Younis et al., 1989). The most predictive morphological criteria for successful in vitro maturation (IVM) and embryo development continue to be an intact cumulus cells and homogenous cytoplasm (Brackette and Zuelke, 1993). However, in order to increase the number of oocytes available for in vitro maturation and fertilization, it may be possible to use denuded oocytes with normal cytoplasm recovered in substantial numbers from buffalo ovaries (Totey et al., 1993).

Competence of any blastocysts resulting after IVM, IVF and IVC will be affected by factors in the culture environment during production of mbryos. Some work comparing media for use in bovine IVM used rates of blastocysts formation to discriminate between media (Krisher and Bavister, 1998). A wide variety of epigenetic factors, including ions, energy substrates, amino acids, vitamins, growth factors, cytokines and hormones play a role in early embryonic development (Gordon, 1994). Recent comparison of several commercially available complex chemically defined media showed that TCM-199 was superior to RPMI-1640 (Gliedt et al., 1996). Other reports suggested that a high percentage of blastocysts developed in Menezo's B2 Compared to TCM-199 medium (Stojkovic et al., 1998). In addition, there are no reports, which demonstrates that culture media can affect the development of buffalo embryos.

The role of gonadotropin in the in vitro maturation of oocytes has been demonstrated. Resumption of meiosis was improved when gonadotropins were added to the culture medium in cattle (Sanbuissho and Threlfalt, 1989) and buffalo (Totey et al., 1993). However, no available literature could be found comparing the type of gonadotropin used in IVM medium of buffalo oocytes.

Embryo development from buffalo oocytes matured and cultured in vitro are lower than those in cattle indicating that optimizing IVM, IVF and IVC remains a challenge. The present study was designed to investigate factors affecting IVP of buffalo embryos, including oocyte quality, type of culture media and the effect of gonadotropin supplementation to maturation medium.

MATERIALS AND METHODS

Collection of ovaries

Ovaries were collected at a local abattoir (El-Monibe, Giza, Egypt) within 20-30 min after slaughter of buffalo cows, and transported in saline solution (0.9 % NaCl) at 25- 35°C within 2-3

h. In the laboratory, ovaries were washed 3 times in modified Dulbeco's phosphate buffered saline (mPBS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Non-atretic antral follicles (2-6 mm diameter) were aspirated with an 18-gauge needle connected to a 10-ml disposal syringe. The contents were pooled in a sterile 10-ml conical tube and allowed to settle for 15 min. After settling part of the follicular fluid was slowly aspirated and the sediments mixed with mPBS (Gibco, Grand Island, USA) plus 3 mg/ml bovine serum albumin (BSA, Sigma, USA) and transferred into a 10 cm sterile culture dish and searched for oocytes under stereomicroscope (28-30x).

Experiment 1

In vitro maturation

This experiment was set to study the effect of oocyte quality on the in vitro production of buffalo embryos. The aspirated/collected oocytes were washed three times in IVM medium. According to the number of cumulus cell layers and ooplasm morphology, oocytes were divided into 3 groups: (1) Good, included oocytes surrounded by compact multi-layers (3-6) of cumulus cells and homogenous ooplasm; (2) Fair, oocytes had 1-2 less compact layers of cumulus cells; and, (3) oocytes with expanded cumulus cells or denuded oocytes were classified as Poor. According to their groups, oocytes were cultured in 35-mm polystyrene culture dish (Falcon, USA). Maturation medium consisted of CR1aa medium supplemented with 5% estrus cow serum (ECS), 10 µg/ml FSH (Denka Phar. Co. Kawasaki, Japan), 50 µg/ml gentamicin (Sigma, USA), 5 mM/ml taurine (Wako, Japan) and covered with mineral oil (Sigma, USA).

Sperm capacitation and in vitro fertilization

One 0.5ml straw of frozen semen was thawed in a water bath at 37°C for 30 sec. Spermatozoa were washed twice by centrifugation in CR1aa medium supplemented with 2.5 mM/ml caffeine sodium benzoate (Sigma, USA) and 10 µg/ml heparin (Shimizu Phar. Co, Shimizu, Japan). After washing, the sperm pellet was suspended in 2 ml CR1aa medium supplemented 10 µg/ml heparin, 5 mg/ml BSA plus 5 mM/ml taurine and the sperm concentration adjusted to 1 x 10^6 /ml. One-hundred µl aliquot of the sperm suspension was placed into a 35 mm polystyrene culture dish and covered with warm mineral oil. After maturation, oocytes were washed 3 times in the same sperm suspension medium, and then 15-20 oocytes transferred into the sperm suspension droplet and cultured under 5% CO_2 at 38.5°C for 5 h in humidified air.

In vitro culture

After fertilization, oocytes were washed twice in IVC medium, then in vitro cultured in CR1aa medium supplemented with 10% superovulated cow serum (SCS), 100 ng/ml insulin (Wako, Japan), 5 mM/ml taurine and 50 µg/ml gentamicin. The supposedly fertilized oocytes were cultured in 4-well culture dish (Nunclon, Denmark) under 5% CO_2 at 38.5°C for 8 days, according to Suzuki et al. (1999). Approximately 20-25 oocyte were placed in each well. Cleavage and embryo development rates (based on morphological characters) were checked on Day 3 and 8 using an inverted microscope. In this experiment, a very low rate of embryos was developed to the blastocyst stage (2-3%). The blastocysts data was excluded from the analyses.

Experiment 2

This experiment was carried out to evaluate the effect of culture media on cleavage rate and development of IVF buffalo embryos. Oocytes with a compact layers of cumulus cells were washed 3 times in IVM medium then cultured for maturation in:(i) CR1aa + 5 mM/ml taurine (Rosenkrans and First, 1994); (ii) CR2aa medium (Wang et al., 1997); (iii) TCM-199 (Gibco BRL, Life Technology, UK)or (iv) MEM (Gibco BRL, Life Technology, UK) and (v) RPMI-1640 (Gibco BRL, Life Technology, UK). All maturation media were supplemented with 5% ECS, 50 µg/ml gentamicin and 10 µg/ml FSH. In all groups, IVF was performed in CR1aa medium as previously mentioned in Experiment 1. For IVC, oocytes in each group were cultured in the same medium used for IVM, supplemented with 5% ECS, 50 µg/ml gentamicin and 100 ng/ml insulin. Checking cleavage and embryo development rates was carried out also in Experiment 1.

Experiment 3

This experiment was designed to elucidate the effect of the addition of gonadotropins to the maturation medium on cleavage rate and development of IVF buffalo embryos. Oocytes with compact dense layers of cumulus cells were washed 3 times in CR1aa medium. Oocytes were classified into 3 groups: (i) Group 1, served as control and was without gonadotropin; (ii) Group 2, had IVM medium supplemented with 10 µg/ml FSH; and (iii) Group 3, had IVM medium supplemented with 10 IU/ml PMSG (Folligon, Invtervet, Netherlands). Maturation medium consisting of CR1aa medium supplemented with 10% ECS, 50 µg/ml gentamicin and 5 mM/ml taurine. In vitro fertilization, in vitro culture, counting cleavage rate and embryo development was carried out as in Experiment 1.

Statistical analysis

Data were statistically analyzed using "Chi-square" according to Snedecor and Cochran (1980).

RESULTS

The effects of oocyte quality on cleavage and developmental rates of IVF buffalo embryos are shown in Table 1. Cleavage rate in Good and Fair quality oocytes was significantly higher ($P<0.01$) than with poor oocytes. In addition, the proportion of embryos that developed to the morula stage was higher ($P<0.01$) in Good than in Fair oocytes. Embryo development did not go beyond the 8-16 cell stage for poor quality oocytes .

The cleavage rate, proportions of embryos that developed to morulae, blastocyst and hatched blastocyst, and those arrested at the 2-16 cell stage among different media used for IVM, IVF and IVC of buffalo oocytes are presented in Table 2. Maturation, fertilization and culture of buffalo oocytes in CR1aa medium produced higher ($P<0.05$) cleavage rate than CR2aa medium (Fig. 1). Difference were significantly higher ($P<0.01$) in CR1aa than TCM-199, MEM or RPMI-1640. The proportion of transferable embryos in term of numbers of morulae/or blastocyst was significantly higher ($P<0.01$) for embryos cultured in CR1aa and CR2aa media when compared with TCM-199 or MEM. Embryos cultured in RPMI-1640 were arrested at 2-16 cell stage, indicating that this medium was not suitable for IVP of buffalo embryos.

Table 1: Effect of oocyte quality on cleavage rate (%) and development of IVF buffalo embryos.

Oocyte quality	No. Oocytes examined	Cleavage rate (%)	No. (%) Embryos development			
			2-cell	4-cell	8 to 16-cell	Morulae
Good	142	99(70)[a]	21(21)	12(12)	3(3)	63(64)[a]
Fair	174	107(61)[a]	33(31)	40(37)	14(13)	20(19)[b]
Poor	147	20(14)[b]	8(40)	10(50)	2(10)	0.0

a,b: Different superscripts in the same column differ significantly ($P<0.01$)

Table 2: Effect of culture media on percentage (%) of cleavage rate and development of IVF buffalo embryos.

Medium	No. oocytes	Cleavage rate (%)	Embryo development (%)			
			2 to 16-cell	Morulae	Blastocysts	H. blastocyst
CR1aa	142	102(72)[a]	27(26)[c]	46(45)b	16(21)[a]	8(8)
CR2aa	190	104(78)[b]	22(21)[c]	51(52)[a]	9(18)[b]	11(11)
TCM 199	158	51(32)[c]	24(47)[c]	14(27)[c]	10(20)[a]	3(6)
MEM	124	48(39)[c]	24(50)[c]	16(33)[c]	8(17)[a]	0.0
RPMI-1640	102	9(9)[d]	9(100)[a]	0.0	0.0	0.0

a, b and b,c different superscripts in the same column differ significantly (P<0.05)
a, d different superscripts in the same column differ significantly (P<0.01)

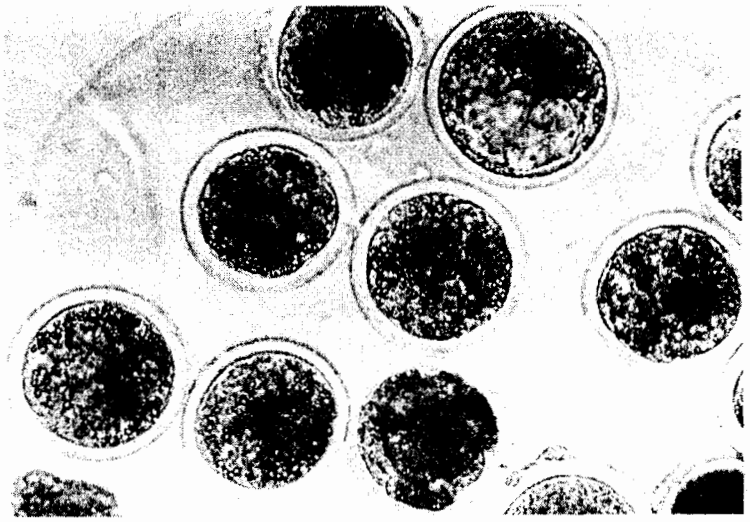

Fig. 1: Buffalo embryo developed up to the blastocyst stage in CR199 medium (400 x)

The effect of gonadotropins on cleavage rate and development of IVF buffalo embryos are presented in Table 3. Cleavage and developmental rates up to the morula stage, blastocysts and hatched blastocysts were significantly higher (P<0.01) when FSH or PMSG were added to the maturation medium compared with non-treated control medium . There was, no difference between FSH and PMSG.

Table 3: Effect of gonadotropins on the percentage (%) of cleavage rate and development of IVF buffalo embryos.

Group	No. Oocytes	Cleavage rate (%)	No. (%) Embryo development			
			2 to 16-cell	Morula	Blastocyst	H. blastocyst
Control	223	28(13)[b]	28(100)[b]	0.0	0.0	0.0
FSH	214	159(74)[a]	63(40)[a]	54(34)	33(21)	9(6)
PMSG	198	136(69)[a]	64(47)[a]	39(29)	27(20)	6(4)

a, b different superscripts in the same column differ significantly (P<0.01)

DISCUSSION

In the present study, data showed that good quality buffalo oocytes surrounded by multi-layers of compact investment with a homogenous ooplasm had a significantly higher cleavage, and developmental rates up to the morula stage compared with oocytes of fair or poor quality. This finding identifies the essential role of cumulus cells in promoting normal cytoplasmic maturation of oocytes necessary for fertilization, cleavage and subsequent development of IVF of buffalo embryos. Our results are similar to those previously reported for buffalo oocytes (Suzuki et al., 1992; Nandi et al., 1998). However, the obtained results disagree with those in which cumulus cells were found to have no influence (Behalova and Greve, 1993), or negative influence on fertilization (Hawk et al., 1992) of bovine oocytes. The mechanism by which oocytes quality can affect fertilization, cleavage and development is not clearly identified. The presence of cumulus cell surrounding the oocytes is essential to facilitate the transport of nutrients and signals into and out of the oocytes (Moor and Seamark, 1986). The cumulus cells improve fertilization rate in cattle by specific and non-specific ways, first by providing a capacitation-inducing mechanism, and secondly by facilitating the interaction between capacitated spermatozoa and the zona pellucida surface (Goud et al., 1998). In addition, De Loose et al. (1989) found that the ultrastructural characterization varied between oocytes with a complete follicle cells investment and those with less investment.

Different media have been investigated for maturation and culture of bovine oocytes with variable results (Gliedt et al., 1996). The present study

showed that IVM, IVF and IVC of buffalo oocytes in CR1aa and CR2aa media produced higher cleavage and embryo developmental rates than TCM-199 or MEM. Moreover, the number of transferable embryos was higher for oocytes cultured in CR1aa or CR2aa media than TCM or MEM, respectively. CR1aa and CR2aa media may contain some components which are beneficial for buffalo embryos to develop to the morula and blastocyst stages. In this respect, the addition of glutamine, taurine and glycine to the culture medium has proven beneficial effect to developing bovine embryos (Takahashi and Kanagawa, 1998). The amino acids could be acting as energy substrates, pH regulator or as a pool for denovo protein synthesis (Rosenkrans and First, 1994). However, our results disagree with that recorded by Wang et al. (1997) they found that there was no difference in the rate of embryo development between CR2aa and TCM-199. Also, Rose and Bavister (1992) observed that MEM-alpha, TCM-199 and RPMI-1640 media were better able to support normal embryo development in vitro. Oocytes matured in medium leading to poor developmental competence have depressed levels of glycolysis at the completion of maturation, although oxidative metabolism remains unchanged when oocytes matured in medium leading to good development to the blastocyst stage. The reduced level of glycolysis may reflect reduced activity of the pentose phosphate pathways, which plays an important role in meiotic maturation of bovine oocytes (Krisher and Bavister, 1998). The present study showed that culture of buffalo oocytes in RPMI-1640 medium produced low rate of cleavage and embryo development, indicating that this medium may not be suitable for in vitro production of buffalo embryos. These results indicated that not all chemical components in complex medium were necessary for in vitro embryo development and, that the selection of medium may be empirical (Bavister, 1995).

In the present work in vitro maturation of buffalo oocytes in CR1aa medium supplemented with either FSH or PMSG increased the cleavage rate and development of IVF buffalo embryos compared with non supplemented control medium. These findings parallel to those previously reported for bovine oocytes (Wang and Niwa, 1995), and for buffalo oocytes (Chauhan et al., 1996), FSH or PMSG enhance oocyte maturation as reflected by greater proportion of matured and inseminated oocytes developing to the blastocysts. Gonadotropin stimulation lead to the generation of a positive factors that acted on the oocyte to override the inhibitory influence and induce germinal vesicle break down (Downs, 1993). Therefore, cAMP-dependent protein kinase activity regulated by cumulus cells following FSH-stimulation may play a role in the complex mechanism of chromatin condensation leading to meiotic resumption in bovine oocytes (Tatemoto and Terada, 1998). The lack of difference be-

tween FSH and PMSG groups indicate that both hormones can be used sucessfuly in the enhancing of maturation, cleavage and embryo development rates of IVP buffalo embryos.

In conclusion, in vitro maturation of good quality buffalo oocytes in CR1aa or CR2aa media in the presence of FSH or PMSG produced significantly higher cleavage and development rates of IVF buffalo embryos up to the morulae and blastocyst stage.

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