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Effect of epidermal and insulin-like growth factor-1 on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in simple serum free media DMEM and Ham's F-10

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ABSTRACT

The object of this study was to identify the possible effects of epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes (COC's) matured and fertilized *in vitro* in two serum free media Dulbecco's modified Eagles medium (DMEM) and Ham's F-10. Oocytes were matured *in vitro* for 24 h either in DMEM or Ham's F-10 with no supplements (control) or in DMEM or Ham's F-10 media supplemented with EGF, IGF-1 or with both EGF and IGF-1. After 24 h of *in vitro* maturation, oocytes were inseminated with sperm prepared in BO medium supplemented with 10 μg ml^{-1} heparin and 0.5% BSA. In Experiment 1 the proportion of oocytes showing maximum cumulus expansion was significantly higher ($P<0.01$) in DMEM medium supplemented with EGF plus IGF-1 at a rate of 51.2% versus the control at a rate of 18.0%. In Ham's F-10 media a significantly higher ($P<0.01$) proportion of oocytes showed maximum expansion when the medium was supplemented with EGF and IGF-1 at a rate of 52.9% versus 19.4% for the control. The same oocytes, when fixed and assessed for nuclear maturation, showed that a significantly higher ($P<0.01$) number of oocytes had reached M-II in both DMEM and Ham's F-10 supplemented with EGF and IGF-1 at a rate of 78.0% and 83.5% versus a rate of 43.0% and 46.3% in their respective controls. In Experiment 2, 24 h after insemination a significantly higher ($P<0.01$) proportion of oocytes were fertilized in both

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DMEM and Ham's F-10 supplemented with both EGF and IGF-1 at a rate of 45.2% and 48.6% versus a rate of 14.6% and 15.0% in their respective controls. These results lead to the following conclusions: (i) supplementation of both DMEM and Ham's F-10 with both EGF and IGF-1 improves cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes; (ii) both serum free media DMEM and Ham's F-10 seem to have the same effect on cumulus expansion, nuclear maturation and fertilization of buffalo oocytes.

Key words: buffalo, epidermal growth factor, insulin-like growth factor, oocyte

Introduction

Recent studies have stressed the need for the use of chemically defined serum-free media for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and subsequent development of follicular oocytes *in vitro*. Amongst the growth factors studied as additives in such media, a combination of epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) appears to be an optimum combination. EGF is a mitogenic factor which has the ability to stimulate the proliferation of ovarian granulosa cells (GOSPODAROWICZ and BIRDWELL, 1977; MAY et al., 1987). Many reports have reported that EGF contributes to the promotion of oocyte maturation (DOWNS et al., 1988; DOWNS, 1989; SANBUISSHO et al., 1991), germinal vesicle breakdown (GVBD), polar body formation (DAS et al., 1991) and cleavage of the oocytes (COSKUN et al., 1991). However, several reports have shown that growth factors had no effect on embryonic development *in vitro* (CARO et al., 1987; WOOD and KAYE, 1989; COLVER et al., 1991). Several previous studies have shown that addition of IGF-1 to culture media *in vitro* promotes maturation of oocytes (HARPER and BRACKETT, 1993; LORENZO et al., 1994; RIEGER et al., 1995) and also affects their subsequent development *in vitro* (HERRLER et al., 1992; PALMA et al., 1997), while a few have shown that addition of IGF-1 had no effect on meiotic maturation, fertilization or embryonic development of oocytes *in vitro* (GRUPEN et al., 1997; GULER et al., 2000). EGF and IGF-1 in combination have been shown to act synergistically and to accelerate the cumulus expansion and the progression of meiosis (LORENZO et al., 1994; PUROHIT, 2001; SAKAGUCHI et al., 2002). This study was designed to evaluate the effect of EGF and IGF-1, singly or in combination, in two serum-free media DMEM and Ham's F-10 on cumulus expansion, nuclear maturation and fertilization *in vitro* of buffalo COC's.

Materials and methods

All reagents and media were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EGF from mouse submaxillary glands (Code E-4127) was used. Ovaries were obtained from an abattoir and were transported to the laboratory in PBS (Dulbecco) at 39 ^{o}C within 2 h. Oocytes were aspirated from antral follicles (2-8 mm in diameter) using an 18-gauge needle attached to a 10 ml disposable syringe. The COC's were washed five times in Hepes buffered washing medium (Dulbecco's modified Eagle's medium (DMEM) or Ham's F-10 medium +0.25 mM sodium pyruvate + penicillin 100 IU ml^{-1} and streptomycin 50 µg ml^{-1}).

Oocytes with homogenous and evenly granulated cytoplasm and 3-4 or more layers of cumulus cells were placed into drops (100 µl) of maturation medium under paraffin oil, and cultured in 35 mm Petri dishes at 39 ^{o}C under an atmosphere of 5% CO_2 in air, 95% humidity for 24 h. The oocytes (five per drop) were matured either in Hepes buffered DMEM or Ham's F-10 media with the addition of sodium bicarbonate, penicillin 100 IU ml^{-1} and streptomycin 50 µg ml^{-1} (control) or control with addition of test substances. Growth factors were added to the serum-free maturation media as described above. There were four different treatments in both the media: control, EGF (10 ng ml^{-1}), IGF-1 (50 ng ml^{-1}) and EGF plus IGF-1 (10 and 50 ng ml^{-1}), respectively.

Sperm preparation and in vitro fertilization (IVF). Thawed frozen thawed buffalo bull semen was prepared for IVF using BO medium as per method described previously (TOTEY et al., 1992). Briefly, thawed frozen semen from a single Murrah buffalo bull preserved in 0.5 ml straws was used. One 0.5 ml semen straw was emptied into a centrifuge tube. Twelve ml of working BO medium was added and the contents were gently mixed. The tube was centrifuged for 5 minutes at 1800 rpm. The supernatant was discarded and the sperm pellet was redissolved in the same quantity of BO medium. The procedure was repeated. After discarding the supernatant the sperm pellet was dissolved in BO capacitation medium (BO working medium with 0.5% BSA fatty acid free) to give a final concentration of 3x10^{6} sperms ml^{-1}. Prepared sperm was incubated in a CO_2 incubator at 38.5 ^{o}C, 5%

CO_2 for 2-3 h before further use. The matured oocytes were washed with BO medium containing 0.5% BSA. Prepared sperm was added into the droplets containing matured oocytes in a Petri dish to give a final concentration of $1x10^6$ sperms ml^{-1}. The dish was placed into a CO_2 incubator at 39 ^oC, 5% CO_2 for 24 h.

Fertilization was assessed after 24 h of sperm-oocyte co-incubation. Oocytes were freed of cumulus cells by repeated pipetting or vortexing for 1 min and were then fixed in acetic methanol (1:3) and stained with 1% orcein in 45% acetic acid. Oocytes were considered fertilized when they had either a sperm head in the vitellus with metaphase-II (M-II) chromosomes, swollen sperm head with M-II or male and female pronuclei. Oocytes that were seen at germinal vesicle, metaphase I or degenerate were considered as arrested.

Experiment 1. The effects of individual or a combination of growth factors in two different maturation media on cumulus expansion and nuclear maturation of oocytes were examined. DMEM and Ham's F-10 with no extra supplements served as the control medium. Growth factors were added to both media separately. Selected COC's were matured randomly in any of the media. After 24 h of *in vitro* culture the oocytes were examined to evaluate cumulus cell expansion as per method described by LORENZO et al. (1994). Briefly, a subjective scoring system was used in which 0 indicated no detectable response; + indicated the minimum observable response and +++ indicated the maximum degree of expansion, where all layers of cumulus cells expanded, even those closest to the oocyte.

After evaluation of cumulus expansion the oocytes were freed of surrounding cumulus cells by treatment with hyaluronidase 200 Uml^{-1} and vortexing for 1 min. The oocytes were then placed in the centre of an area delineated by two paraffin wax (10:1) bars on a clean, grease-free glass slide. The oocytes were compressed gently with a coverslip to hold and were fixed for 24 h by placing the slides in acetic acid: ethanol (1:3, v/v). The fixed oocytes were stained by placing a drop of aceto-orcien (1% orcein in 45% acetic acid) for 2 min followed by washing under tap water.

Nuclear maturation was evaluated and classified as germinal vesicle (GV), metaphase I (MI) (including anaphase 1 and telophase-1), metaphase–II (M-II) and degenerate as described by LORENZO et al. (1994). Oocytes from each replicate (n = 8) of each treatment were fixed, stained and evaluated as a group.

Experiment 2. COC's were matured for 24 h in the four different treatments in each media. Oocytes were then fertilized as previously described. After 24 h of sperm oocyte incubation the oocytes from each replicate within the same treatment were fixed and stained and evaluated for fertilization at x400 as a group.

Statistical analysis. The degree of cumulus expansion and metaphase II stage were used as end-point parameters for assessing the effect of growth factors on expansion and maturation of bovine oocytes *in vitro*. Cumulus expansion at the end of culture period was assigned a numerical value corresponding to the degree of expansion achieved, where minimum response (+) equals 1, moderate response (++) equals 2 and maximum response (+++) equals 3. These numerical values were subjected to analysis of variance. The data (n = 618 COC's) were analysed by replication (n = 8) and treatment (n = 4) comparisons between least square means (LS ± SEM) by t test. The arcsine transformed data of the proportion of oocytes reaching M-II stage in Experiment 1 or fertilized in Experiment 2 were compared by ANOVA and Duncan multiple range test.

Results

A total of 1323 oocytes of culturable grade recovered from 985 buffalo ovaries were used in this study.

In Experiment 1 (n = 618), the respective proportion of COC's that attained cumulus expansion (% expansion +++ and LS Means ± SEM) in the four different treatments in DMEM medium were 18.05% and 1.62 ± 0.4 (control), 41.4% and 4.0 ± 0.72 (EGF), 38.3% and 3.87 ± 0.71 (IGF-1) and 51.2% and 5.25 ± 0.7 (EGF+IGF-1), respectively. The respective figures in Ham's F-10 medium were 19.4% and 1.62 ± 0.34 (control), 40.5% and 3.87 ± 0.58 (EGF), 36.4% and 3.5 ± 0.65 (IGF-1) and 52.9% and 5.62 ± 1.01 (EGF+IGF-1) (Table 1).

Table 1. Effect of different media and growth factors on cumulus expansion of buffalo oocytes matured *in vitro*

Treatment groups	Total number of oocytes*	Cumulus expansion responses				
		0 (%)	+ (%)	++ (%)	+++ (%)	LSM ± SEM
DMEM (Control)	72	56.94	15.27	9.72	18.05	1.62 ± 0.40^{b}
Ham's F-10 (Control)	67	53.73	14.92	11.94	19.40	1.62 ± 0.34^{b}
DMEM + EGF	81	18.66	17.34	22.67	41.40	4.0 ± 0.72^{ab}
Ham's F-10 + EGF	77	22.78	17.72	18.98	40.50	3.87 ± 0.58^{ab}
DMEM + IGF-1	75	29.62	17.28	14.81	38.27	3.87 ± 0.71^{ab}
Ham's F-10 + IGF-1	79	32.46	14.28	16.88	36.40	3.5 ± 0.65^{ab}
DMEM + EGF + IGF-1	82	17.07	12.19	19.51	51.22	5.25 ± 0.70^{a}
Ham's F-10 + EGF + IGF-1	85	14.12	11.76	21.17	52.95	5.62 ± 1.01^{a}

Cumulus expansion – 0 = no detectable response; + = minimum observable response equals 1; ++ = moderate response equals 2; +++ = indicates the maximum degree of cumulus expansion equal 3

Values within column with different superscripts are significantly different ($P<0.01$)

* = data pooled from 8 independent experiments

Table 2. Effect of different media and growth factors on the nuclear status of *in vitro* matured buffalo oocytes

Treatment groups	Total number of oocytes*	Nuclear stages			
		DG	GV	M I	M II
DMEM (Control)	72	20.83 ± 0.48	19.44 ± 0.51	16.67 ± 0.47^{a}	43.05 ± 0.51^{b}
Ham's F-10 (Control)	67	19.40 ± 0.60	19.40 ± 0.37	14.92 ± 0.40^{ab}	46.30 ± 0.45^{b}
DMEM + EGF	81	23.45 ± 0.29	7.4 ± 0.63	12.34 ± 0.60^{ab}	56.79 ± 0.45^{ab}
Ham's F-10 + EGF	77	16.88 ± 0.38	11.68 ± 0.50	7.79 ± 0.40^{ac}	63.64 ± 0.44^{ab}
DMEM + IGF-1	75	18.66 ± 0.33	10.67 ± 0.47	9.33 ± 0.58^{ac}	61.14 ± 0.56^{ab}
Ham's F-10 + IGF-1	79	27.78 ± 0.25	6.33 ± 0.25	3.79 ± 0.47^{ac}	67.08 ± 0.59^{ab}
DMEM + EGF + IGF-1	82	17.07 ± 0.25	6.09 ± 0.21	2.5 ± 0.31^{bc}	78.04 ± 0.71^{a}
Ham's F-10 + EGF + IGF-1	85	14.12 ± 0.20	2.5 ± 0.00	0.00 ± 0.51^{c}	83.52 ± 0.85^{a}

Values within column with different superscripts are significantly different ($P<0.01$), ($P<0.05$)

* = data pooled from 8 independent experiments

DG = degenerate; GV = germinal vesicle; M I = Metaphase I (including metaphase I, anaphase I and telophase I); M II = Metaphase II

Values are percentage ± SEM

Table 3. Fertilization rates of *in vitro* matured buffalo oocytes in different media under different treatment groups

Treatment groups	Total number of oocytes*	Nuclear stages		Percentage of total oocytes fertilized
		Arrested	M II	
DMEM (Control)	75	64.00 ± 0.37^{ab}	21.34 ± 0.49	14.67 ± 0.30^{b}
Ham's F-10 (Control)	80	60.00 ± 0.53^{ab}	25.00 ± 0.61	15.00 ± 0.50^{b}
DMEM + EGF	84	40.47 ± 0.58^{b}	38.09 ± 0.62	21.42 ± 0.55^{b}
Ham's F-10 + EGF	92	68.47 ± 0.59^{a}	14.13 ± 0.62	17.39 ± 0.51^{b}
DMEM + IGF-1	79	48.10 ± 0.55^{b}	24.05 ± 0.56	27.84 ± 0.52^{ab}
Ham's F-10 + IGF-1	82	37.80 ± 0.44^{b}	26.82 ± 0.76	35.36 ± 0.34^{ab}
DMEM + EGF + IGF-1	104	34.62 ± 0.50^{b}	20.19 ± 0.49	45.19 ± 0.51^{a}
Ham's F-10 + EGF + IGF-1	109	29.35 ± 0.53^{b}	22.02 ± 0.51	48.62 ± 1.05^{a}

Values within column with different superscripts are significantly different (P<0.01).
* = data pooled from 8 independent experiments
M II = Metaphase II; Arrested = includes degenerated (DG), germinal vesicle (GV) stage and metaphase I (M I).
Values are percentage ± SEM

Treatment with EGF or IGF-1 resulted in a non-significantly higher incidence of cumulus expansion. However, treatment with EGF+IGF-1 resulted in a significantly higher (P<0.01) incidence of cumulus expansion compared with control.

The respective proportion of COC's that reached M-II stages in the four treatments over the two media DMEM and Ham's F-10 media were 43.05% and 46.3% (control), 56.7% and 63.6% (EGF), 61.1% and 67.0% (IGF-1) and 78.0% and 83.5% (EGF+IGF-1), respectively (Table 2). In both DMEM and Ham's F-10 medium, supplementation with both EGF and IGF-1 resulted in a significantly higher proportion (P<0.05) of oocytes which reached M II compared to the control medium. No significant effect was observed after treatment with EGF or IGF-1 individually compared with the control. The maturation medium apparently had no significant effect on the proportion of oocytes that reached M-II stage compared to control.

In Experiment 2 (n = 705) the proportion of oocytes that were fertilized when previously matured in DMEM or Ham's F-10 medium

supplemented with both EGF and IGF-1 was significantly higher ($P<0.01$) compared to their respective controls. No significant effect was observed in fertilization rate when the oocytes had been previously matured in media supplemented with EGF or IGF-1 alone compared with control (Table 3). No significant differences were found between the 2 media for the four treatments in the proportion of oocytes that were fertilized.

Discussion

The role of serum on meiosis resumption and oocyte maturation (FUKUI et al., 1982; XU et al., 1986; YOUNIS et al., 1989) has been demonstrated. However, several studies have recommended the use of serum-free medium for *in vitro* fertilization (MENEZO et al., 1984; NAGAE et al., 1991; TAKAGI et al., 1991) because serum quality varies from batch to batch and serum may contain materials toxic to the cell culture (OGAWA et al., 1987). It is difficult to define components already contained in the serum. Growth factors such as EGF and IGF-1 have a positive effect on oocyte maturation in various species (PARK and LIN, 1993; KOBAYASHI et al., 1994; LONERGAN et al., 1996; GRUPEN et al., 1997; PALMA et al., 1997; GULER et al., 2000; SAKAGUCHI et al., 2002).

The present study demonstrated that both growth factors enhanced cumulus expansion in buffalo cumulus oocyte complexes, but the results were marked when both EGF and IGF-1 were combined. Previous studies have shown that EGF promotes cumulus expansion (KOBAYASHI et al., 1994; LORENZO et al., 1994; GRUPEN et al., 1997; GULER et al., 2000) contrary to IGF-1, do (LORENZO et al., 1994; GRUPEN et al., 1997; GULER et al., 2000), probably either because IGF does not act via cumulus cells, or it interferes with the production of an expansion factor produced by the oocyte (BUCCIONE et al., 1990). However, HAINAUT et al. (1991) postulated that maturation with IGF-1 is initiated upon activation of the membrane receptor for this growth factor and requires tyrosine dephosphorylation of p 34, the kinase component of maturation promoting factor (MPF). IGF-1 has been shown to act on the development of bovine embryos indirectly, via granulosa cells (PALMA et al., 1997), the IGF-1 receptors being located in the plasma membrane of granulosa cells (ADASHI et al., 1988) and that IGF-1 stimulates

the proliferation and differentiation of these cells (SPICER et al., 1993). However, LORENZO et al. (1996) obtained high nuclear maturation with IGF-1 in rabbit oocytes.

The proportion of oocytes that reached M-II stage at the end of Experiment1 or those that were fertilized at the end of Experiment 2 during the present study were significantly higher ($P<0.01$) in both DMEM and Ham's F-10 medium supplemented with a combination of EGF and IGF-1 compared to their respective controls. This finding indicates an additive action between the growth factors used and suggests that their actions are combined under conditions *in vitro*. Similar findings have been previously reported (LORENZO et al., 1994; LORENZO et al., 1996).

The present study demonstrated that there were no differences in the oocyte maturation or fertilization rates between the two media DMEM and Ham's F-10 under serum-free conditions. When the same supplements were added individually to both the media, the results were comparable. Various previous reports have shown that there is no effect of media on the nuclear maturation, fertilization and subsequent development of bovine (FUKUI et al., 1982; WANG et al., 1997) pig (WANG et al., 1991; LONG et al., 1999; PETERS et al., 2001) or buffalo (TOTEY et al., 1992) oocytes.

In conclusion, both EGF and IGF-1 enhance cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in serum free media DMEM and Ham's F-10 and when combined their effects are additive. The culture medium DMEM or Ham's F-10 has little effect on the cumulus expansion, nuclear maturation or fertilization of buffalo COC's.

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SAŽETAK

Cilj istraživanja bio je utvrđivanje mogućeg učinkaa epidermalnog čimbenika rasta (EGF) i inzulinu-sličnog čimbenika rasta 1 (IGF-1) na ekspanziju kumulusa, dozrijevanje jezgre i oplodnju bivoljih jajnih stanica dozrelih i oplođenih *in vitro* u dvjema različitim hranilištima bez dodatka seruma: Eaglovom hranilištu preinačenom po Dulbeccu (DMEM) i Hamovom hranilištu F-10. Jajne stanice su dozrele *in vitro* tijekom 24 sata u DMEM ili Hamovom F-10 hranilištu bez dodatka i uz dodatak EGF i IGF-1. Nakon 24 sata dozrijevanja *in vitro*, jajne stanice su osjemenjene spermom pripremljenom u BO hranilištu uz dodatak 10 mg ml^{-1} heparina i 0,5% goveđeg serumskog albumina. U prvom pokusu je udio jajnih stanica koje su sasvim ekspandirale bio signifikantno viši ($P<0,01$) u DMEM hranilištu obogaćenom s EGF i IGF-1 i iznosio je 51,2%, u odnosu na kontrolne skupine gdje je ekspandiralo samo 18,0% jajnih stanica. U Hamovom hranilištu F-10 znatno više ($P<0,01$) jajnih stanica pokazalo je potpunu ekspanziju kada je bilo obogaćeno s EGF i IGF-1 (52,9% u odnosu na 19,4% u kontrolnoj skupini). Jajne stanice bile su zatim fiksirane te im je određeno dozrijevanje jezgre. Značajno veći ($P<0,01$) broj jajnih stanica dosegao je M-II stadij u DMEM i Hamovom F-10 hranilištu uz dodatak EGF i IGF-1 (78,0% i 83,5% u odnosu na 43,0% i 46,3% u kontrolnim skupinama). U drugom pokusu, 24 sata nakon inseminacije signifikantno viši ($P<0,01$) udio jajnih stanica bio je oplođen i u DMEM i Hamovom F-10 hranilištu uz dodatak EGF i IGF-1 (45,2% i 48,6% u odnosu na 14,6% i 15,0% u kontrolnim skupinama). Ovi rezultati upućuju na sljedeće zaključke: (1) dodatak EGF i IGF-1 u DMEM i Hamovom F-10 hranilištu poboljšali su ekspanziju kumulusa, dozrijevanje jezgre i oplodnju bivoljih jajnih stanica; (2) čini se da oba hraništa imaju isti učinak na ekspanziju kumulusa, dozrijevanje jezgre i oplodnju bivoljih jajnih stanica.

Ključne riječi: bivol, epidermalni čimbenik rasta, čimbenik rasta sličan insulinu, jajna stanica