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EFFECT OF EPIDERMAL AND INSULIN-LIKE GROWTH FACTOR-1 ON NUCLEAR MATURATION AND EMBRYO DEVELOPMENT OF BUFFALO OOCYTES IN VITRO

(With 5 Tables)

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تآثيرإضافة عوامل النمو الشبيه بالإنسولين وبالإبيدرمل على معدل نضوج وإخصاب ونمو بويضات الجاموس معمليا

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تهدف الدراسة الحاليه إلي دراسة تآثيرإضافة عوامل النمو (عامل النمو الشبية بالإنسولين-1 أو عامل النمو الشبية بالإبيدرمل) بتركيزات مختلفه إلي ميديا النضوج المحدد تركيبها الكيميائى على معدل نضوج وإخصاب ونمو بويضات الجاموس معمليا. تم تجميع البويضات من الحويصلات المتوسطة الحجم (2 الى 8 مللى) وبعد تقييم البويضات بناء على شكل الخلايا الوسادية المحيطة بالبويضة وكذالك شكل السيتوبلازم للبويضة. تم إنضاج البويضات لمدة 24 ساعة فى ميديا النضوج المحدد تركيبها الكيميائى بدون أى إضافات كمجموعة ضابطة أو مجموعة مضاف إليها عامل النمو الشبية بالإبيدرمل بتركيزات (5،10 ، 20 نانوجرام/مللى) أو عامل النمو الشبية بالانسولين بتركيزات (50،25 ، 100 نانوجرام/مللى) كلا على حده أو معا. وبعد ذلك تم إخصاب البويضات الناضجة بسائل منوى مجمد بعد معالجته بالهيبارين لمدة 18 ساعة وفى نفس ظروف الإنضاج. وبعد تقييم معدل الإخصاب تم زراعة البويضات المخصبة معمليا لمدة سبعة ايام وملاحظة النمو حتى طور البلاستوسيست. ولقد أظهرت النتائج أن إضافة 5 نانوجرام/مللى عامل النمو الشبية بالإنسولين- 1 و 50 نانوجرام/مللى عامل النمو الشبية بالإبيدرمل معا إلي ميديا النمو قد أدى إلي زيادة معنويه كبيرة فى معدل النضوج والإخصاب ومعدل النمو إلي الطور التوتى وطور البلاستوسيست (83,48 ، 62,00 ، 29,12 ، 22,87% على التوالى) بالمقارنة بالمجموعه الضابطه (32,75، 61,93، 7,25 ، 2,80% علي التوالي). من خلال نتائج الدراسه الحاليه يمكن أن نستنتج أن عوامل النمو لها تأثيرإيجابي على معدل النضوج والإخصاب والنمو المعملي بناء علي الجرعة المؤثرة. كما أن إضافه 5 نانوجرام/مللى من عامل النمو الشبية بالإنسولين-1 أو 50 نانوجرام/مللى من عامل النمو الشبيه بالإبيدرمل معا إلي ميديا النمو له تأثير تعضيدى مما يؤدى إلي زيادة معنوية

كبيرة لمعدل النضوج والإخصاب المعملى ومعدل النمو إلي الطور التوتى وطور البلاستوسيست.

SUMMARY

The present study was aimed to investigate the possible effects of epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) on nuclear maturation, fertilization and embryo development in vitro of buffalo cumulus oocyte complexes (COC's). Oocytes were matured in vitro for 24 h in a defined SOF media with no supplements (control) or in SOF media supplemented with EGF (5, 10 and 20 ng/ml), IGF-1 (25, 50, 100 ng/ml) or with a combination of EGF and IGF-1. After 24 h of in vitro maturation, oocytes were inseminated with sperm prepared in S-TALP medium supplemented with 10 μg/ml heparin. Embryos were evaluated for cleavage and development to the morula and blastocyst stages. The current results revealed that, addition of a combination of 5 ng/ml EGF plus 50 ng/ml IGF-1 to the maturation medium significantly increased ($P<0.01$) the in vitro maturation, fertilization rate and embryo development to the morula and blastocyst stages (83.48± 2.27, 62.00±3.19, 29.12±4.13 and 22.87±2.26%, respectively) as compared with the control (61.93±4.09, 32.75±2.77, 7.25±1.45 and 2.80±1.42%, respectively). In conclusion, the current results inferred that EGF and IGF-1 had a positive effect on buffalo ooctes maturation, fertilization and subsequent embryo development in a dose dependent trend. Supplementation of maturation medium with a combination of 5 ng/ml EGF and 50 ng/ml IGF-1 enhanced the in vitro maturation, fertilization and embryo development.

Key words: *Buffalo, epidermal growth factor, insulin-like growth factor, in vitro fertilization.*

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. In general, chemically defined culture media afford development of lower blastocyst yields (Sirisathien

and Brackett, 2003). To improve blastocyst development, several common growth factors have been added to embryo culture media as they are known to be involved in embryo development (Baştan *et al.,* 2010). 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 (May *et al.,* 1987). Many reports have reported that EGF contributes to the promotion of oocyte maturation (Sanbuissho *et al.,* 1991), germinal vesicle breakdown (GVBD), polar body formation (Das *et al.,* 1991) and cleavage of the oocytes (Coskun *et al.,* 1991). Moreover, several previous studies have shown that addition of IGF-1 to culture media in vitro promotes maturation of oocytes (Harper and Brackett, 1993; Rieger *et al.,* 1995) and also affects their subsequent development in vitro (Herrler *et al.,* 1992; Palma *et al.,* 1997). 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). Despite their actions on early embryonic development, growth factors are not routinely included in embryo culture medium. The lack of exposure to the proper growth factor milieu may be one reason why embryos produced in vitro differ from their counterparts derived in vivo (Block *et al.,* 2007). However, a few reports 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). Therefore, the current study was designed to evaluate the effect of EGF and IGF-1, singly or in combination, in serum-free SOF medium on in vitro nuclear maturation, fertilization and embryo development of buffalo COC's.

MATERIALS and METHODS

Oocyte selection and in vitro maturation:

Ovaries were obtained from an abattoir and were transported to the laboratory in PBS at 35^{o}C within 2 h. Oocytes were aspirated from medium follicles (2-8 mm in diameter) using an 18-gauge needle attached to a 10 ml disposable syringe. Only

oocytes having a dense cumulus cell mass and homogeneous cytoplasm were selected. Oocytes were washed 3 times with Dulbecco's phosphate-buffered saline. The selected oocytes were cultured in 35 mm Petri dishes at 39^{o}C under an atmosphere of 5% CO_2 in air, 95% humidity for 24 h for maturation. The oocytes were matured in SOF media with the addition of sodium bicarbonate, penicillin 100 IU ml/1 and streptomycin 50 μg ml/1 (control) or with addition of test substances (growth factors). After 24 hours, nuclear maturational status was assessed by aceto-orcien stain as described by Lorenzo *et al.* (1994).

Sperm preparation and in vitro fertilization (IVF):

Three straws of frozen semen were thawed in a water bath at 38^{o}C for 30 sec. After thawing, the most motile spermatozoa were separated by swim up technique in sperm-TALP medium containing 6 mg/ml bovine serum albumin, for 1h (Parrish *et al.*, 1986). The uppermost layer of the medium containing the most spermatozoa was collected. The selected spermatozoa were washed twice by centrifugation at 2000 rpm for 10 minutes. The sperm pellet was re-suspended in the fertilization TALP (F-TALP) medium containing 10 μg/ml heparin. The prepared sperm was incubated in a CO_2 incubator at 38.5^{o}C, 5% CO_2 for 2 h before further use. The matured oocytes were washed with F-TALP medium and the prepared sperm was added into the droplets containing matured oocytes to give a final concentration of 2 X10^{6} sperm cells/ml. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 hour. At the end of gametes co-incubation, some of inseminated oocytes were freed of the attached cumulus cells, fixed in acetic acid- ethanol (1:3), stained with 1% aceto-orcein stain and examined under phase-contrast microscope (X 400) for assessing the in vitro fertilization rate according to Totey *et al.* (1992).

In vitro culture:

Presumptive zygotes were denuded from cumulus cells and the extra spermatozoa by gentle pipetting and transferred, into SOFaa culture medium (SOF with 1 mM glutamine, 1% MEM nonessential amino acids and 1% MEM essential amino acids) and covered with mineral oil for 7- days at 38.5 ^{o}C in an atmosphere of 5% CO_2 in air with maximum humidity. The

proportional of cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded at 5-7 days post-insemination according to Totey *et al.* (1992).

Experiment 1:

The effect of different growth factor concentrations, individually or in a combination, on nuclear maturation of buffalo oocytes were examined. Maturation medium with no extra supplement served as control. Growth factors were added to maturation medium at different concentrations, IGF-1 (25, 50, 100 ng/ml, respectively) or EGF (5, 10 and 20 ng/ml, respectively). Selected COC's were matured randomly in the media of different treatments. After 24 h the oocytes were examined to evaluate in vitro maturation rate.

Experiment 2:

COC's were matured for 24 h in the best concentrations of the growth factors resulted from experiment 1, singly or in combination. The matured oocytes were then fertilized and cultured in vitro to evaluate fertilization and embryo development rates in relation to each treatment.

Statistical analysis:

All data were analyzed by using *Costat Computer Program, Version 3.03* copyright (1986) *Cottort Software*, and were compared by the least significant difference least (LSD) at 1% and 5% levels of probability.

RESULTS

Data presented in Table 1 revealed that, fortification of the maturation medium with different concentrations of IGF-1 improved the in vitro maturation rate compared to the control in a dose-dependent trend. Addition of 50 ng/ml IGF-1 to the maturation medium improved ($P<0.01$) significantly the in vitro maturation rate (72.04± 3.45 %) as compared with the control (51.48± 4.55 %).

Table 1: Effect of different concentrations of insulin-like growth factor 1 on the in vitro maturation rate.

Treatment	No. of oocytes	No. of matured oocytes	Maturation rate
IGF-1 25ng/ml	75	50	66.79± 1.95 [a]
IGF-1 50 ng/ml	54	39	72.04± 3.45 [a]
IGF-1 100 ng/ml	72	39	54.17± 1.17 [b]
Control	78	40	51.48± 4.55 [b]

IGF-1: insulin-like growth factor 1
Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Results presented in Table 2 revealed that, supplementation of the maturation medium with different concentrations of EGF improved the in vitro maturation rate compared to the control in a dose-dependent trend. Addition of 5 ng/ml EGF to the maturation medium increased (P<0.01) significantly the in vitro maturation rate (79.35± 3.42 %) as compared with the control (64.73± 2.99 %).

Table 2: Effect of different concentrations of epidermal growth factor on buffalo oocytes maturation rate in vitro.

Treatment	No. of oocytes	No. of matured oocytes	Maturation rate
EGF 5ng/ml	83	66	79.35± 3.42 [a]
EGF 10 ng/ml	81	60	74.15± 3.77 [ab]
EGF 20 ng/ml	86	56	65.15± 1.68 [b]
Control	76	49	64.73± 2.99 [b]

EGF: Epidermal growth factor
Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Data regarding the effect of replenishing of the maturation medium with the best concentration of the growth factors resulted from the previous experiments either singly or in combination is presented in table 3. The current results revealed that, when growth factors are combined their effects are additive.

Table 3: Effect of insulin-like growth factor 1 and /or epidermal growth factor on the in vitro maturation rate.

Treatment	No. of oocytes	No. of matured oocytes	Maturation rate
IGF-1 50ng/ml	54	41	76.03± 1.99 [a]
EGF 5 ng/ml	57	44	77.07± 1.14 [a]
IGF-1 0ng/ml+ EGF 5 ng/ml	73	61	83.48± 2.27 [a]
Control	71	44	61.93±4.09 [b]

IGF-1: insulin-like growth factor 1 EGF: Epidermal growth factor
Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Results regarding the effect of augmentation of the maturation medium with 50 ng/ml IGF-1 and/or 5 ng/ml EGF on the in vitro fertilization rate are presented in table 4. The current results revealed that, addition of IGF-1 and EGF in combination to the maturation medium had a positive effect on the in vitro fertilization rate (P<0.01) as compared with the control (62.00±3.19 *vs*. 32.75±2.77).

Table 4: Effect of insulin-like growth factor 1 and /or epidermal growth factor on the in vitro fertilization rate

Treatment	No. of oocytes	Penetration rate	Fertilization rate	Abnormal fertilization
IGF-1 50ng/ml	73	54 (73.97±1.16) [a]	32 (45.16±3.57) [b]	13 (17.91±1.91)[ab]
EGF 5 ng/ml	81	55 (67.87±2.54) [a]	36 (44.44±1.86) [b]	13 (16.86±2.80) [ab]
IGF-1 50ng/ml+ EGF 5 ng/ml	76	55 (72.59±2.50) [a]	47 (62.00±3.19) [a]	9 (11.77±1.92) [b]
Control	71	50 (70.74±2.73) [a]	23 (32.75±2.77) [c]	16 (22.26±1.73) [a]

IGF-1: insulin-like growth factor 1 EGF: Epidermal growth factor
Values with different superscript letters in the same columns are significantly different at least (P<0.05).

Likewise, data presented in Table 5 revealed that, combination of IGF-1 and EGF resulted in a significant increase (P<0.01) in the cleavage rate and embryo development to the

morula and blastocyst stages (46.56±3.48, 29.12±4.13 and 22.87±2.26%, respectively) as compared with the control (28.83±1.66, 7.25±1.45 and 2.80±1.42%, respectively).

Table 5: Effect of insulin-like growth factor 1 and /or epidermal growth factor on the in vitro embryo development rate.

Treatment	No. of oocytes	Cleavage rate	Morula stage	Blastocyst stage
IGF-1 25ng/ml	61	24 (39.81±2.65) [a]	10 (16.59±1.53) [b]	6 (9.96±1.97) [b]
EGF 5 ng/ml	70	29 (41.85±3.09) [a]	13 (18.93±2.63) [b]	8 (11.49±1.51) [b]
IGF-1 50ng/ml+ EGF 5 ng/ml	75	35 (46.56±3.48) [a]	22 (29.12±4.13) [a]	17 (22.87±2.26) [a]
Control	69	20 (28.83±1.66) [b]	5 (7.25±1.45) [c]	2 (2.80±1.42) [c]

IGF-1: insulin-like growth factor 1 EGF: Epidermal growth factor
Values with different superscript letters in the same columns are significantly different at least ($P<0.05$).

DISCUSSION

The role of serum on meiosis resumption and oocyte maturation (Younis *et al.,* 1989) has been demonstrated. However, several studies have recommended the use of serum-free medium for in vitro fertilization (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. The present study demonstrated that EGF and IGF-1 enhanced the in vitro maturation, fertilization and embryo development in a dose-dependent trend, but the results were marked when both EGF and IGF-1 were combined. These results were in accordance with previous studies having shown that EGF and IGF-1 have a positive effect on oocyte maturation (Kobayashi *et al.,* 1994). Supplementation of EGF resulted in a higher proportion of nuclear maturation and normal fertilization rates compared to the control in a dose-dependent manner (Im and Park, 1995). The dose dependent effect of EGF supplementation in increasing the proportion of oocytes reaching nuclear maturation was evident only up to 20 ng/ml. As pointed out by

Harper and Brackett (1993), EGF in serum is possibly one of the undetermined components contributing to enhanced oocyte maturation. In the present study, EGF supplementation increased significantly the cleavage, and embryo development rate compared with the control. These results are in consistent with, Lonergan *et al.* (1996); Baştan *et al.* (2010) who reported that blastocyst development was significantly improved for oocytes matured in the presence of EGF.

Moreover, one growth factor that modifies embryonic physiology is insulin-like growth factor-1 (IGF-1). In the present study, IGF-1 treatment tended to increase the proportion of ooytes that matured, fertililized and developed to the blastocyst stage. Similar results were also observed in several studies (Lorenzo *et al.*, 1996; Block *et al.*, 2007). The effect of IGF-1 on embryo development may be partly explained by differences in culture systems since there are reports that the actions of IGF-1 to stimulate embryonic development depend upon culture conditions. IGF-1 has been shown to act on the development of bovine embryos indirectly, via granulose 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). Moreover, IGF-1 can reduce the proportion of blastomeres that are apoptotic (Sirisathien and Brackett, 2003), alter the abundance of some developmentally important genes (Block *et al.*, 2007), and increase cellular resistance to heat shock (Jousan and Hansen, 2007). Also, IGF-1 can increase development of bovine embryos to the blastocyst stage (Block and Hansen, 2007) and can increase blastocyst cell number (Sirisathien *et al.*, 2003).

Furthermore, the proportion of oocytes that matured, fertilized or developed in vitro were significantly higher in the defined SOF medium supplemented with a combination of EGF and IGF-1 compared to their respective controls. These results were in accordance with previous studies having shown that EGF and IGF-1 have a positive effect on embryo development in various species (Lorenzo *et al.*, 1996; Kumar and Purohit, 2004; Baştan *et al.*, 2010). This finding indicates a synergistic action between the growth factors used and suggests that their actions are collective under in vitro conditions.

In conclusion, the current results inferred that EGF and IGF-1 had a positive action on buffalo ooctes maturation, fertilization and subsequent embryo development in a dose dependent trend. Supplementation of maturation medium with a combination of 5 ng/ml EGF and 50 ng/ml IGF-1 enhanced the in vitro nuclear maturation, fertilization and embryo development.

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