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Influence of Epidermal Growth Factor with Cysteamine on *in -Vitro* Buffalo Embryo Development

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FOR IMPROVING embryo development in buffalo, two experiments were conducted. The first one was carried out to evaluate the different concentrations (0, 5, 25, 50 ng/ml) of epidermal growth factors (EGF) on developmental competence of buffalo oocytes. The selected oocytes were cultured in the four concentrations of EGF. The embryo cleavage rate was significantly higher in oocytes exposed to 5, 25, 50 ng/ml EGF than control. There were no significant difference in four groups (0, 5, 25, 50 ng/ml EGF) in the rate of morula. But, better cleavage and blastocyst rates were observed at 5 ng/ml EGF. In the second experiment, the additive effect of 5 ng/ml EGF with 50 µM cysteamine on maturation and embryo development was studied. Oocytes were collected, matured and cultured in three groups. In the first group, media supplemented with 5 ng/ml EGF + 50 µM cysteamine combination. In the second group, media supplemented with EGF. The third group was supplemented with cysteamine. There was a significant increase in cleavage rate in combination group than EGF ($P< 0.05$) and cysteamine ($P< 0.01$) groups. But there was no significant difference in cleavage rate between EGF and cysteamine. The morula percentage was nearly similar in the three groups. But blastocyst rate was significantly ($P<0.05$) higher in combination group than cysteamine. Thus better cleavage and blastocyst rate were observed at combination group. It is concluded that, the addition of 5 ng/ml EGF in buffalo oocytes during in vitro culture was the best concentration for embryo developmental competence. Higher cleavage and blastocyst rate were achieved by combination of epidermal growth factor and antioxidant.

Keywords: Buffalo oocytes, EGF, Cysteamine, Embryo development, Culture media.

There were many growth factors as insulin growth factors (IGFs), epidermal growth factor (EGF), transforming growth factor α, β and activin acted as

regulators for modulate follicular development, granulosa cell proliferation, decreased apoptosis and promoted follicular antrum formation (Mtango *et al.*, 2002 and Purohit*c et al.*, 2005). The EGF was first discovered as one from EGF family proteins (Dreux *et al.*, 2006). It had a mitogenic effect in a variety of species such as cattle (Lorenzo *et al.*,1994, Kobayashi *et al.*, 1994 and Reiger *et al.*, 1995), pigs (Reed *et al.*, 1993, Ding and Foxcroft, 1994), rodents (Das *et al.*,1992 and Demeestere *et al.*, 2005), buffalo (Chauhan *et al.*, 1999 and Kumar & Purohit, 2004), sheep (Grazul-Bilska *et al.*, 2003, Shabankareh and Zandi, 2010), dog (Bolamba *et al.*, 2006), rabbit (Lorenzo *et al.*,1996), cat (Merlo *et al.*, 2005) and humans (Das *et al.*,1991 and Gomez *et al.*,1993).

EGF had a positive effect during *in vitro* maturation by stimulating the ovarian granulosa cells proliferation (May *et al.*,1987), growth of preantral follicles (Gutierrez *et al.*,2000), promoting oocyte maturation (Sanbuissho *et al.*,1991), germinal vesicle breakdown, polar body formation (Das *et al.*, 1991) and cleavage of oocytes (Coskun *et al.*,1991). EGF stimulated DNA synthesis in cumulus cells (Lonergan *et al.*, 1996 and Khamsi & Armstrong, 1997), induced proteoglycan synthesis (Das *et al.*, 1991)**,** production of tissue plasminogen activator and urokinase plasminogen activator by cumulus cells which stimulated oocyte maturation (Park *et al.*, 1999).

Cysteamine was a low molecular weight thiol compound that might reduce cysteine to cystine which enhanced oocyte glutathione synthesis (Issels *et al.*, 1988) which protected the cell from oxidative damage (Wang & Ballatori, 1998, Hammond *et al.*, 2001 and Deleuze & Goudet, 2012), improved the formation of male pronucleus, protein, DNA synthesis and reduction of disulphides (Kim *et al.*, 2004). In buffalo, cysteamine supplementation was reported to improve nuclear maturation rates (Singhal *et al.*, 2009) by increasing GSH synthesis (Gasparrini *et al.*, 2003) and improve male pronucleus formation (Anandi *et al.*, 2008). Furthermore, cysteamine increased cleavage rates following IVM (Singhal *et al.*, 2009) and subsequent embryonic development in vitro (Ocampo and Ocampo, 2015).

Based on the above knowledge, this work aimed to improve buffalo embryo development by using growth factor as epidermal growth factor and prevent oxidative stress by cysteamine addition.

Material and Methods

Experimental Designs

Experiment 1: Evaluation the effect of different concentrations of EGF on developmental competence of buffalo oocytes.

EGF was added to maturation and culture media at different concentrations (0, 5, 25, 50 ng/ml), oocytes incubated in CO_2 incubator at 38°C for 5-7 day. The optimum concentration which achieved higher cleavage, morula and blastocyst rate was recorded.

Experiment 2: The effect of combination of EGF and cysteamine on developmental competence of buffalo oocytes.

Three groups of buffalo oocytes matured and cultured in CO_2 incubator at 38°C for 5-7 days. Oocytes was cultured in media supplemented with both 5 ng/ml EGF + 50 µM cysteamine (Group I), 5ng/ml EGF (Group II). 50 µM cysteamine (Group III). Cleavage, morula and blastocyst rate were recorded.

Chemicals

Chemicals for *in vitro* maturation as fetal calf serum and tissue culture medium (TCM-199) were obtained from Gibico (Grand Island, New York, USA). Cysteamine (M 6500), epidermal growth factor (E1257) and chemicals for *in vitro* fertilization were obtained from Sigma Chemical Company.

Oocyte recovery

Ovaries were collected from Cairo abattoir within 2 h of slaughter and transported to the laboratory in saline (0.9% NaCl) containing antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin) maintained at 30°C. After washing the ovaries in phosphate- buffered saline (PBS), oocytes were aspirated from 2 to 5 mm follicles with a 20-gauge needle containing PBS with 0.3% bovine serum albumin (BSA) and antibiotics (100 µg/ml streptomycin sulfate and 100 IU/ml penicillin).

In vitro oocyte maturation

The maturation was carried out as previously described (Mahmoud, 2001). The selected oocytes were cultured in medium consisted of TCM-199, 10% calf serum, and 50 µg/ml gentamycin. The droplets were covered with mineral oil and were pre-incubated for a minimum 2 h at 38.5 ºC 5% CO_2 in air with 95% humidity. The oocytes were added to droplets and incubated for 24hr.

In vitro fertilization and culture

Spermatozoa were treated as described previously by Niwa and Ohgoda (1988). Briefly, two straws of frozen buffalo semen were thawed in a water bath at 35–37 ºC for 1 min. The spermatozoa were washed by centrifugation (800×g for 10 min) in BO medium (Brackett and Oliphant, 1975) without BSA containing10 µg/ml heparin and 2.5 mM caffeine. The pellets were diluted with BO medium containing 20 mg/ml bovine serum albumin to adjust the concentration of spermatozoa to 12.5×10^6 sperm/ml. Matured oocytes were washed in BO medium containing 10 mg/ml BSA and were introduced into 100 µl droplets of sperm suspension under paraffin oil, the spermatozoa and oocytes were co-cultured in the same culture conditions (5% CO_2, 38.5 ºC, 95% humidity) for 5 h under. After that, the oocytes were washed in TCM-199 to remove attached spermatozoa. Groups of 10–20 oocytes were replaced with previously prepared co-culture 100 µl droplet consisting of TCM-199+10% serum. Cleavage was recorded after 72 hr of culture (day 0=day of insemination)

and the embryos developing to the morula and blastocyst stages were assessed at days 5 and 7, respectively.

Statistical analysis

Our results were tabulated to indicate the mean values of the studied parameters and their standard errors. Data were analyzed by ANOVA using SPSS version 18.0, statistical software. Comparison of means was performed by Duncan's Multiple Range Test. Differences were considered to be significant at $P < 0.05$ level.

Results

In the first experiment

Oocytes were matured and cultured in different concentrations of EGF and fertilization output was evaluated. Data in Table 1 indicated that the cleavage rate was significantly ($P< 0.05$) higher in oocytes treated with 5, 25, 50 ng/ml EGF than control. While the embryo cleavage rate was not significantly differ between 25 ng/ml and 50ng/ml of EGF. The better cleavage rate was observed at concentration 5 ng/ml EGF.

The mean percentage of morula after treated with different concentrations (0, 5, 25, 50 ng/ml) of EGF was illustrated in Table 1. There was no significant difference in oocytes treated by 5, 25, 50 ng/ml EGF and control group in the percent of morula. The morula percent was 45.94, 46.38, 48.0 and 44.9 for control, 5, 25, 50 ng/ml EGF, respectively.

The blastocyst rate after treated with different concentrations (0, 5, 25, 50 ng/ml) of EGF was recorded in Table 1. The blastocyst rate was significantly increased ($P< 0.05$) in 5 ng/ml EGF than control. At the same time, there were no significant differences between control and other concentrations of 25 and 50 ng/ml. Better blastocyst rate was observed at 5 ng/ml .

TABLE 1. The effect of different EGF concentrations on embryo developmental rate of buffalo oocytes (Mean± S.E).

Epidermal growth factor concentrations	No. Inseminated oocytes	Cleavage No. (%)	Morula No. (%)*	Blastocyst No. (%)*
Control (0)	84	45 (54.31 ± 2.6)^{b}	38 (45.94 ±2.4)^{a}	7 (8.37 ± 0.4)^{b}
5 ng/ml EGF	87	53 (62.41± 2.7)^{a}	40 (46.38± 3.1)^{a}	11 (12.52 ± 1.1)^{a}
25 ng/ml EGF	97	58 (61.15 ± 1.1)^{ab}	47 (48.00 ± 2.0)^{a}	12 (11.82 ± 1.0)^{ab}
50 ng/ml EGF	98	55 (56.77 ± 1.3)^{ab}	43 (44.9 ± 1.8)^{a}	9 (9.89 ± 1.6)^{ab}

*Percent from total inseminated oocytes

No.= number

$^{a, b}$ Values within same column without common superscripts differ ($p<0.05$).

In the second experiment

The combination effect between epidermal growth factor and cysteamine on developmental competence of buffalo oocytes was evaluated.

Data regarding the combination effect between 5 ng/ml of EGF and 50 µM cysteamine on cleavage, morula and blastocyst rates was illustrated in Table 2. There was a significant increase in cleavage rate in combination group than EGF ($P< 0.05$) and cysteamine ($P< 0.01$) groups. But, there were no significant differences in cleavage rate between EGF and cysteamine treated groups.

The morula percentages were 50.43 %, 46.73 %, 42.21 % for combination, EGF and cysteamine groups, respectively. There were no significant difference between combination and EGF group in morula rate. Morula percentage was nearly similar in EGF and cysteamine groups. While, the morula percentage was significantly ($P< 0.05$) higher in combination than cysteamine group.

The mean proportion of blastocyst rates were illustrated in Table 2. The blastocyst rate was significantly ($P< 0.05$) higher in combination group than cysteamine. But, there was non-significant difference between combination group and EGF group. Blastocyst rate was nearly similar in EGF and cysteamine groups. Thus, better blastocyst rate was observed at combination of EGF and cysteamine group.

TABLE 2. The effect of EGF and cysteamine combination on developmental competence of buffalo oocytes (Mean ± S. E).

Treatment groups	Total No. of inseminated oocytes	Cleavage No. (%)	Morula No. (%)*	Blastocyst No. (%)*
EGF + Cysteamine	88	64 (72.88 ± 1.1)[a]	43 (50.43 ±2.5)[a]	15 (17.70 ± 1.9)[a]
EGF	131	83 (64.88 ± 2.8)[b]	60 (46.73 ±1.6)[ab]	16 (13.07±1.5)[ab]
Cysteamine	98	60 (60.51 ± 2.5)[b]	42 (42.21 ±2.6)[b]	12 (12.32 ± 0.5)[b]

*Percent from total inseminated oocytes

No.= number

[a , b] Values within the same column differ significantly ($P<0.05$- $P<0.01$).

Discussion

The first aim in the present study analyzed the effect of different concentrations of EGF on buffalo embryo developmental rate. In our result, the embryo cleavage rate was significantly higher in oocytes treated with 5, 25 and 50 ng/ml of EGF concentrations than control. While, there were no significant difference in four groups (0, 5, 25, 50 ng/ml EGF) in the rate of morula. But, at 5 ng/ml EGF, the better blastocyst rate was observed. So, the highest cleavage and blastocyst rate were achieved at concentration 5 ng/ml EGF.

Our result was in accordance with Sirisathien *et al.* (2003) and Thongkittidilok *et al.* (2015) who observed that oocyte competence and blastocyst developmental rate were improved at concentration 5 ng/ml EGF compared with un supplemented group in bovine and cat, respectively**.** It was suggested that EGF enhanced oocytes developmental competence and blastocyst formation in single-embryo culture system, but did not affect embryo development in cultured groups, this discrepancy might be due to a species-specific response to EGF (Thongkittidilok *et al.,* 2015).

In contrast to our study, many reports stated that oocytes maturation and embryo development rate were increased at concentration 10 ng/ml EGF in several species as bovine (Lonergan *et al.,* 1996 and Mtango *et al.,* 2003), porcine (Abeydeera *et al.*, 1998, Sirotkin *et al.,* 2000 and Mao *et al.*, 2004) and buffalo (Kumar & Purohit, 2004 and Purohit *et al.,* 2005). While, Singhal *et al.* (2009), Kandil *et al.* (2013) and Sadeesh *et al.* (2014) mentioned that the optimum concentration of EGF was 20 ng/ml for buffalo embryo development *in vitro*.

It was suggested that at higher concentrations (40 ng/ml), blastocyst development was reduced (Carpenter & Cohen, 1976 and Sirotkin *et al.,* 2000) due to a phenomenon termed growth factor-induced receptor down regulation. Thus the presence of high concentration of EGF caused a significant down regulation or acceleration of EGF receptors degradation (Beguinot *et al.*, 1984).While in sheep, Ni *et al.* (2015) proved that *in vitro* embryo developmental rate was significantly higher at concentration 50 ng/ml EGF. But, the suitable concentration for mouse embryo development was 1 ng/ml EGF (Merriman *et al.*, 1998).

When TCM-199 was supplemented with optimum concentration of EGF during IVM, cumulus cells expansion were stimulated, percentage of nuclear matured oocytes were increased as well as the proportion of embryos attaining blastocyst stage were increased (Buyalos and Cai, 1994, Lonergan *et al.*, 1996). EGF considered as local regulators for cell proliferation and differentiation (Teruel *et al.,* 2000), might be one of the signaling factors for resumption of meiosis of oocytes (Coskun *et al.,* 1991), promoted follicular antrum formation, and suppressed granulosa cell apoptosis (Mao *et al.,* 2002). Also, EGF stimulated DNA synthesis in cumulus cells (Khamsi and Armstrong, 1997) and stimulated the pattern of proteins neosynthesis (Lonergan *et al.,* 1996). Moreover, Lee and Fukui (1995) observed that the stimulatory effect of EGF existed in post-fertilization bovine embryonic development especially at morulae/early blastocysts than early IVM condition. When bovine blastocysts were cultured, vitrified in media with EGF, higher developmental capacity were observed (Mtango *et al.,* 2003).

On the other hand, some studies stated that the growth factors as EGF had no positive effect on mouse (Wood & Kaye, 1989 and Colver *et al.,* 1991) and bovine (Yang *et al.,*1993 and Keefer *et al.,* 1994) embryonic developmental rate *in vitro*.

In the present work, the additive effect of 5ng/ml EGF and 50 µM cystamine on maturation and embryo development were studied. There was a significant increase in cleavage and blastocyst rates in combination group than EGF and cysteamine groups. But, the morula percentage was nearly similar between three groups.

EGF along with antioxidants increased maturation rate which might be due to assessment of oocyte maturation on the basis of cumulus expansion, not by nuclear maturation (Lorenzo *et al.*, 1994 and Singhal *et al.*, 2009). It was suggested that cysteamine was a better antioxidant than β-mercaptoethanol for *in vitro* embryo production (De Matos *et al.*, 2002). Addition of cysteamine enhanced the glutathione synthesis in maturation medium (De Matos and Furnus, 2000) to improve oocyte maturation by protecting oocytes from oxidative stress (Gasparrini *et al.*, 2003). In the culture medium, cysteamine improved blastocyst production rate and enhanced embryo quality in various species as bovine (Lojkic *et al.*, 2012) and buffalo (Ocampo and Ocampo, 2015). Moreover, it improved transformation efficiency of sperm head nucleus into male pronucleus during fertilization.

In opposite to our result, Singhal *et al.* (2009) mentioned that using 20 ng/ml EGF+ 50 µM cysteamine had no positive effect on oocyte maturation and buffalo embryo development. While, oocyte maturation and embryo development were improved by using 20ng/ml EGF + 100 µM β-mercaptoethanol. Oyamada and Fukui (2004) reported that the combination group had no positive effect on nuclear maturation, but improved cleavage rate of bovine oocytes. The difference between previous studies and our results may be attributed to the concentration of growth factor.

Conclusion

The addition of 5 ng/ml EGF during *in vitro* culture was the best concentration for embryo developmental competence. Higher cleavage and blastocyst rate were achieved by combination of epidermal growth factor and cysteamine in buffalo.

Declaration of interest

The authors declare that there is no conflict of interest.

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تأثير عامل نمو الخلايا اللحائية (EGF) و السيستامين على تطور أجنة الجاموس معمليًا

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أجريت تجربتين لدراسة تحسين تطور أجنة الجاموس معملياً و تهدف التجربة الأولى لتقييم تركيزات مختلفة من عامل نمو الخلايا اللحائية (EGF) وهى صفر و ٥ و ٢٥ و ٥٠ نانوجرام /ميلى على قدرة النمو لبويضات الجاموس و قد تم زراعة البويضات فى الأربعة تركيزات المذكورة و قد وجد أن معدل انقسام الأجنة فى التركيزات ٥ و ٢٥ نانوجرام / ميلى اعلي معنويا عن المجموعة الضابطة و لم تكن هناك اختلافات معنوية بين المجموعات الأربعة عند مرحلة الموريولا ولكن عند مرحلة البلاستوسست كانت الانقسامات أفضل عند تركيز ٥ نانوجرام/ميلى وفى التجربة الثانية تم دراسة التأثير الإضافى للسيستامين بتركيز ٥٠ ميكرو مولر مع ٥ نانوجرام / ميلى EGF على نضج ونمو الأجنة معمليا، تم تجميع ونضج وزراعة البويضات فى ثلاث مجموعات، فى المجموعة الأولى تم إضافة ٥٠ ميكرو مولر سيستامين مع ٥ نانوجرام / ميلى EGF. وفى المجموعة الثانية تم إضافة ٥ نانوجرام/ ميلى EGF. وفى المجموعة الثالثة تم إضافة ٥٠ ميكرو مولر سيستامين وقد وجد أن أفضل معدل انقسامات كانت فى المجموعة الأولى بينما لم تكن هناك فروق معنوية بين الثانية والثالثة وكانت نسبة الموريولا متساوية تقريبا فى الثلاث مجموعات.و كانت نسبة البلاستوسست أفضل فى المجموعة الأولى .

و الخلاصة ان إضافة ٥ نانوجرام/ميلى EGF لبويضات الجاموس أثناء النمو كان أفضل تركيز لنمو الأجنة. و كانت أعلى معدلات الانقسام و نسبة البلاستوسست عند اتحاد٥٠ ميكرو مولر سيستامين مع ٥ نانوجرام/ ميلى EGF

الكلمات الدالة: بويضات الجاموس، عامل نمو الخلايا اللحائية (EGF) ، سيستامين، نمو الأجنة، بيئة الإنماء.