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


*Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, Benha **Department of Animal Reproduction and A.I., National Research Centre, and ***Artificial Insemination and Embryo Transfer Dept., Animal Reproduction Research Institute, Cairo, Egypt.

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 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 (Sanbuisho 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 (Gasparini 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 CO₂ 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 °C for 1 min. The spermatozoa were washed by centrifugation (800xg for 10 min) in BO medium (Brackett and Oliphant, 1975) without BSA containing 10 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 12.5x10\(^6\) 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 °C, 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 different between 25 ng/ml and 50 ng/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).**

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

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).

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total No. of inseminated oocytes</th>
<th>Cleavage No. (%)</th>
<th>Morula No. (%)*</th>
<th>Blastocyst No. (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF + Cysteamine</td>
<td>88</td>
<td>64 (72.88 ± 1.1)$^a$</td>
<td>43 (50.43 ±2.5)$^a$</td>
<td>15 (17.70 ± 1.9)$^a$</td>
</tr>
<tr>
<td>EGF</td>
<td>131</td>
<td>83 (64.88 ± 2.8)$^b$</td>
<td>60 (46.73 ±1.6)$^{ab}$</td>
<td>16 (13.07±1.5)$^{ab}$</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>98</td>
<td>60 (60.51 ± 2.5)$^b$</td>
<td>42 (42.21±2.6)$^b$</td>
<td>12 (12.32 ± 0.5)$^b$</td>
</tr>
</tbody>
</table>

*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 a non-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, 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 (Khamis 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 morulac/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.

References
Presence of β-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenol., 50, 747-756.


(Received 22/8/2016; accepted 24/11/2016)
INFLUENCE OF EPIDERMAL GROWTH FACTOR (EGF) AND THE SISTAMINES ON THE GROWTH OF THE UMBILICAL TISSUE

Ahmed El-Mahroum, Mohamed El-Sheikh El-Tenawy, and Alaa Eldin El-Zanaty

The aim of this study was to investigate the effect of EGF and the histamine and serotonin on the growth of the umbilical tissue. The study was conducted on 50 newborn lambs divided into two groups, each consisting of 25 lambs. The first group was fed with EGF and the histamine, while the second group was fed with EGF and the serotonin. The results showed that the group fed with EGF and the histamine had a significantly higher growth rate than the group fed with EGF and the serotonin. The study concluded that EGF and the histamine have a positive effect on the growth of the umbilical tissue.