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Effect of Epidermal Growth Factors (EGF) on the Maturation and Developmental Competence of Buffalo's Oocytes and Embryo Stages in vitro

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Abstract

The present study aims to study the effects of epidermal growth factors (EGF) supplementations to the in vitro maturation (IVM) medium of buffalo's oocytes on the oocyte maturation rate and embryo development competence. The ovaries were collected from El-Monieb slaughters house and transferred directly to the laboratory for the experiment processing. Excellent and good cumulus oocytes complexes (COCs) were aspirated from 3-8 mm diameter follicles. TCM-199 medium were used for in vitro maturation. In experiment I, COCs were incubated in EGF-containing IVM medium as; 0 (control or CTL), 10, 20 and 50 ng/ml EGF for 24 hrs at 38.5°C in humidified environment; 5% CO_2 and 95% humidity. In experiment II, COCs were incubated in standard in vitro fertilization (IVF) medium with the same concentrations for 24 hrs in the same condition. In experiment III, COCs were incubated in standard in vitro culture (IVC) medium with the same concentrations for 24 hrs in the same condition. According to the expansion of oocytes and attaining the developmental embryo stages of morula and blastocyst, our results have shown that addition of 20 ng/ml of EGF to the IVM medium significantly increased the oocyte maturation and fertilization rates compared to those of CTL (76.96 ± 9.04 vs. 51.3 ± 4.66 and 67.96 ± 2.76 vs. 42.03 ± 4.83 %, respectively) ($P<0.05$). On the other hand, Addition of 20 ng/ml of EGF to the IVM medium significantly increased the fertilization rates compared to other concentrations (10 and 50 ng/ml) (67.96 ± 02.76 vs. 49.43 ± 03.67 and 34.70 ± 01.40 %, respectively). However, the developmental rates to morula and blastocyst stages in response to addition to IVC medium variably increased rather than those of control despite no significance.

Keywords: Bubalus bubalis, Buffalo, EGF, IVM, Oocytes.

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Introduction

Buffalos (*Bubalus bubalis*) are multi-purpose animals for agriculture system as it not only provides milk, meat but also its value for labour and ability to resist environmental temperature, climate, stress and diseases (Ismail et al., 2016). However, buffaloes have low reproductive efficiency and limit the productivity which expressed in long post-partum anestrus period (Singh and Krishan, 1992), delayed age of puberty and low conception rates collectively (Nandi et al., 2002). The essential process for the optimal fertilization and developmental competence after oocytes maturation are resumption and completion of meiosis in mammalian oocytes are (Eppig, 1996). In addition, the nuclear and cytoplasmic maturation of oocytes are involved with the activation of oocytes, fertilization process and embryo development before implantation (Eppig et al., 1994) and it requires sufficient nutrients from follicular fluid and cells (Steeves and Gardner, 1999; Sutton-McDowall et al., 2005) as growth factors and hormones which promote nuclear and cytoplasmic maturation of oocytes (Mattioli et al., 1988; Procházka et al., 2000). Among growth factors, epidermal growth factor (EGF) plays an important role in cell growth, proliferation and differentiation by stimulation of mitosis in various types of cells (Yarden, 2001) and induces resumption of meiosis in mammalian oocytes (Bolamba et al., 2006; Lindbloom et al., 2008).

Hernandez et al. (1988) demonstrated that EGF participates in the regulation of many ovarian functions as a potent mitogen for granulosa cells, a biological amplifier of FSH action in the ovary and considered enhancer for oocyte maturation (Leibfried and First, 1979). The supplementation of maturation medium (TCM-199) with EGF alone during in vitro maturation (IVM) at physiological concentrations stimulates cumulus cell expansion and improves the percentage of oocytes undergoing nuclear maturation (Lonergan et al., 1996). Despite numerous studies performed on the three main phases of in vitro embryo production (IVEP) (IVM, In vitro fertilization (IVF) and In vitro development (IVD), their efficacy is still low, with only 30 to 40% of oocytes developing into blastocysts (Camargo et al., 2006).

From all the above, the specific aim of this study was to assess the effects of EGF to IVM on the development competence of matured buffalo's oocytes, subsequent fertilization and developed embryos.

Materials and Methods

The present study was carried out at the IVF unit, Department of Artificial Insemination and Embryo Transfer, Animal Reproduction Research Institute (ARRI), EL-Haram, Giza, Egypt. All procedures in this study were conducted according to the Animal Ethics Committee of the South Valley University for Veterinary Research, Qena, Egypt.

I. Collection of ovaries:

Three hundred seventy ovaries (370) were collected from apparently healthy buffaloes with normal reproductive tracts and unknown breeding histories were slaughtered in El Monieb, Giza slaughterhouse. The ovaries were collected within 30 minutes after slaughter of the animals. The specimens were placed in a thermos containing normal saline (0.9% NaCl with 100 IU/ml Penicillin and 100 µg/ml Streptomycin) at a temperature of 35°C to preserve the viability of the ovaries during the period of transportation to the lab. The samples were transported to the laboratory within 1hr. The ovaries were

washed several times in a warm 0.9% NaCl solution at 37°C until obtaining clear transparent saline free from blood and then kept in a water bath at 37°C during the oocytes collection. The follicular fluids were aspirated from follicles (3 to 8 mm) using a 16 gauge needle attached to a 10 ml syringe filled with aspiration media (TCM-199). The follicular fluids containing oocytes were pooled in a sterile 50 ml centrifuge tube and were allowed to settle for 30 min at 37 °C. After settling, about 5 ml of the sediment was aspirated and placed in a 10 cm-diameter polystyrene sterile Petri dish. Four hundred and seventy cumulus oocytes complexes (COCs) with compact multilayered cumulus investment and evenly granulated cytoplasm (Grade 1 as described by Chauhan et al., 1998) were selected for in vitro maturation. The oocytes were picked up with a sterile glass pipette under a stereomicroscope and transferred to another dish containing washing medium TCM-199.

II. Experimental design and IVM:

Oocytes with compact multilayered cumulus were divided into three major experiments according to addition of EGF. In experiment I, COCs were incubated in EGF-containing IVM medium as; 0 (control or CTL), 10, 20 and 50 ng/ml EGF for 24 hrs at 38.5°C in humidified environment; 5% CO2 and 95% humidity. In experiment II, COCs were incubated in standard IVF medium as; 0 (control or CTL), 10, 20 and 50 ng/ml EGF for 24 hrs in the same condition. In experiment III, COCs were incubated in standard IVC medium as; 0 (control or CTL), 10, 20 and 50 ng/ml EGF for 24 hrs in the same condition.

The pH of all media was adjusted to 7.4 and all media were filtered through 0.2 µm filter (Pal life Sciences, Ann Arbor, USA) just before use. The cumulus oocyte complexes were washed several times with the IVM medium and groups of 10-15 COCs were placed in 50 µl droplets of the IVM medium, covered with sterilized mineral oil in a 35- mm Petri dish and cultured for 24 hrs under 5% CO2 at 38.5 °C.

III. In vitro fertilization:

For IVF of matured buffalo oocytes, oocytes matured in maturation medium were partially denuded from the surrounding cumulus cells to allow easy penetration of the sperm cells. They were washed twice in pre-warmed fertilization medium to maintain the defined component of the IVF media and IVM oocytes were fertilized in vitro TALP media. The matured oocytes were washed with F-TALP and partially denuded. The frozen semen straws form artificial insemination unit at ARRI, selected by swim up and incubated with caffeine (3.83 mg) for capacitation. About 5-15 matured oocytes were placed in each well of a culture dish containing 75 µl of fertilization media, to which 25 µl of sperm suspension (motile spermatozoa (10 x 106 separated by swim up using sperm TALP medium) were added, they were covered with sterile mineral oil and placed in a CO2 incubator (Forma Scientific, Inc.comp., 35485/NModel) at 38.5 °C for 20-22 hrs (Parrish et al., 1986). At the end of this stage, the penetration rate in which the rate of oocytes penetrated by the sperms, and the subsequent fertilization rate in which the rate of successful fertilization of oocytes were recorded.

IV. In vitro culture:

At the end of sperm-oocyte incubation, prior to the transfer to the IVC droplets, presumed zygotes were washed four times in embryo culture medium

(SOF) and cultured in this medium in a humidified CO2 incubator at 38.5 °C.

The embryo production rate was examined under Stereomicroscope (nanshige, Nikon, SM. DIA, Japan) to record the number of cleaved embryos at 8-16 cells after 94-96 hrs post insemination. Cleavage was recorded after 72 hrs 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.

V. Statistical analysis:

Each experiment was replicated at least three times. Data were presented as mean and standard error (mean ± SEM). Statistical significance was determined by analysis variance (One-Way ANOVA and t-test) by Graph pad Prism, Version 5 for confirm the significance. Statistically significant differences values were set at $P \leq 0.05$.

Results

I. Experiment I: Effect of EGF-maturation medium on the buffalo oocytes maturation rate and embryo stages in vitro:

Ten, 20 and 50 ng/ml of EGF added to the maturation medium showed variable effects on the oocyte maturation rate (Expressed in expansion of the cumulus cells). The former two concentrations increased the maturation rate of the oocyte in vitro while the later concentration did not. Our results have shown that 20 ng/ml of EGF significantly increase in the maturation rate of oocytes ($P<0.05$) rather than other two concentrations compared to the control group (76.96 ± 9.04 vs. 51.33 ± 4.66 %) (Fig. 1A and Fig. 2).

II. Experiment II: Effect of EGF-maturation medium on the fertilization rate in vitro:

Twenty ng/ml of EGF added to the maturation medium tended to significantly increased the penetration and fertilization rates of the oocytes compared to control (65.49 ± 3.83 vs 61.66 ± 2.56 and 67.96 ± 2.76 vs 42.03 ± 4.83 %, respectively) (Fig. 1B).

III. Experiment III: Effect of EGF-maturation medium on the embryo stages in vitro:

Interestingly, in comparison to the control group, EGF increased the incidence of cleavage, morula stage and blastocyst formation and that the concentration 20 ng/ml significantly increased the incidence of cleavage rate (30.76 ± 4.66 vs 61.10 ± 4.85 %, respectively), morula stage (27.76 ± 14.69 vs 49.46 ± 3.64 %, respectively) ($P<0.05$). While, 50 ng/ml of EGF highly increased the formation of morula stage than 20 ng/ml of EGF (61.13 ± 5.56 vs 49.46 ± 3.64 %, respectively). However, 20 ng/ml of EGF tended to significantly increase the formation of blastocyst compared to control (58.90 ± 4.85 vs 50.00 ± 28.86 %, respectively) (Fig. 1C and Fig.3)

Discussion

To enhance *Bubalus bubalis* oocyte maturation rates, it is necessary to modify a suitable oocyte maturation medium with various supplements. The process of meiotic maturation and acquisition of developmental competence determine the ability of the oocytes to undergo successful fertilization, cleavage, and embryonic development. Little information is available on IVM and IVF of buffalo oocytes. In buffaloes, despite a similar maturation rate, a significantly lower cleavage rate was observed in comparison to cattle with 87 vs 94%, and 65 vs 84%, respectively (Gasparrini, 2002; Neglia et al., 2003). Therefore, it is a prompt concern to further improve IVEP so that it

can be widely used for buffalo. These important steps are dependent on a variety of factors that lead to proper nuclear and cytoplasmic maturation (Trounson et al., 2001).

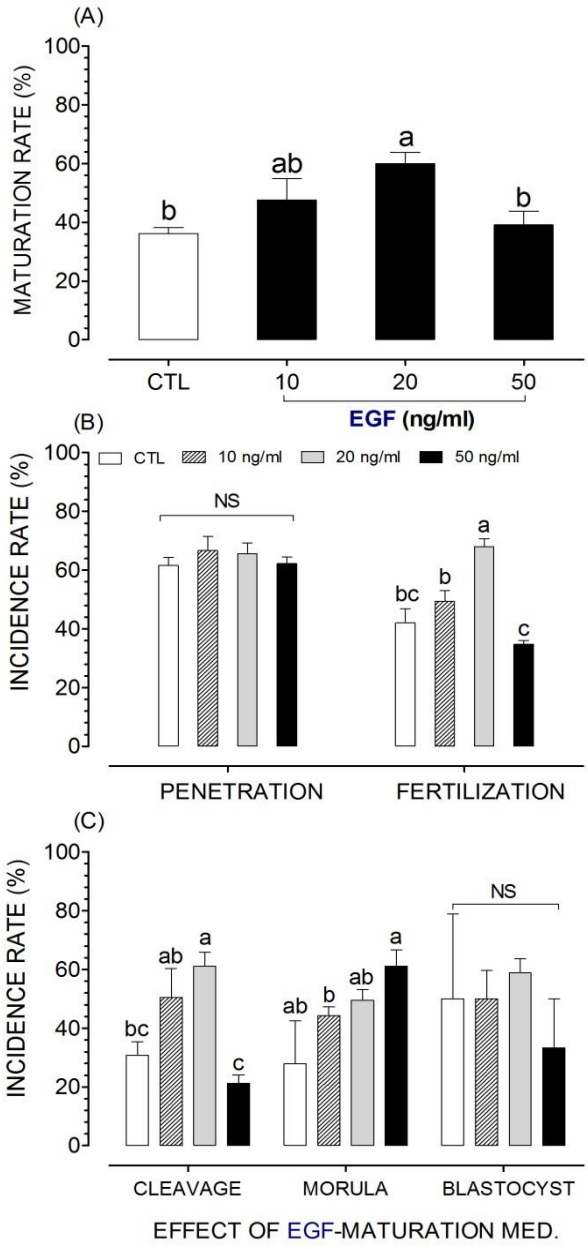

Fig. 1. The oocyte maturation rate, penetration/fertilization and the embryo stages in response to addition of epidermal growth factor (EGF) to the maturation medium in response to the EGF are shown in fig. 1A, B and C, respectively. The EGF maturation medium was used in variable conc. according to the groups of treatment: 0 (vehicle), 10, 20 and 50 ng/ml were used in the control and treated groups. The total number (n) of oocytes used were 47, 46, 48 and 43 in fig. 1A, 34, 36, 38 and 37 in fig. 1B and 29, 37, 40 and 38 in fig. 1C per each respective group. All data were presented as mean ± SEM. The letters on bar (a, b and c) denote significant difference at $P<0.05$.

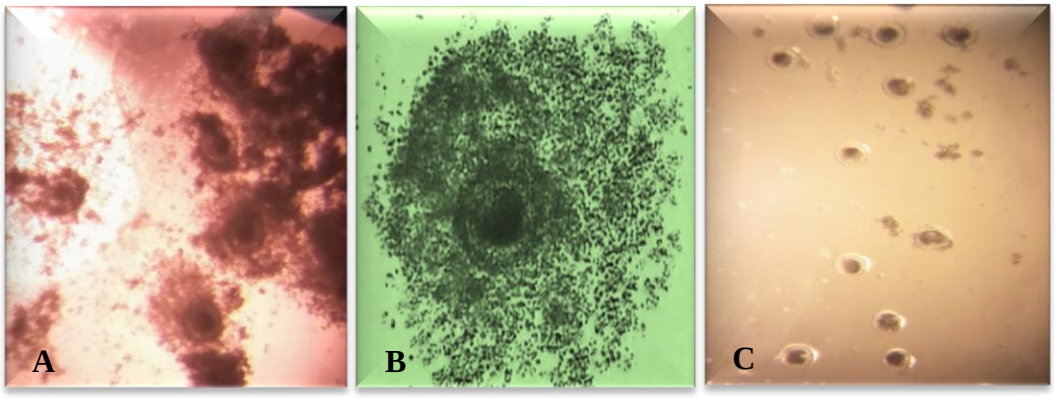

Fig. 2. In vitro matured oocytes. In vitro matured oocytes with expanded cumulus mass (A) and (B) and denuded oocytes after washing three times in F-TALP medium (C).

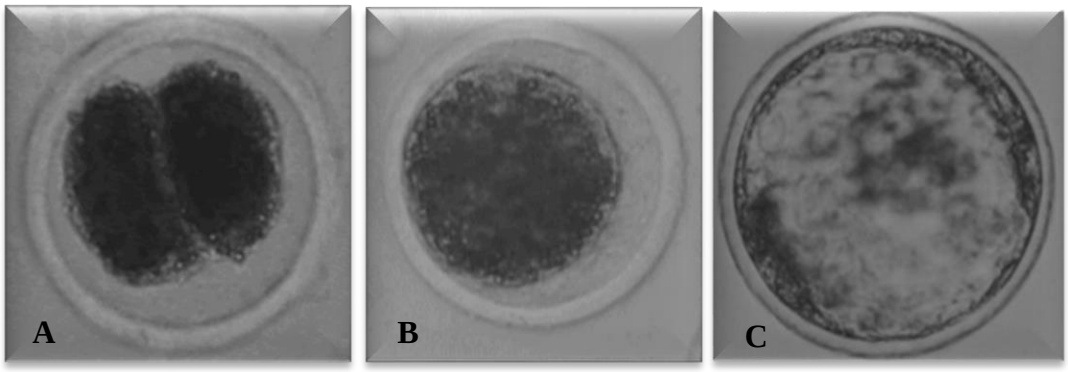

Fig. 3. Evaluation of the cleavage rate, morula and blastocyst formation. Cleaved oocyte with 2 cell stage (A), compact morula (B) and expanded blastocyst (C).

Growth factors as EGF may be represented as local regulators in the coordination of cellular proliferation and differentiation. In that regard, EGF stimulates cellular functions which effect on early development of mammalian embryos (Teruel et al., 2000). The present study demonstrated that EGF enhanced cumulus expansion in buffalo cumulus oocyte complexes. The present study recorded that 20 ng/mL EGF was more beneficial on embryo development than other two examined concentrations (10 and 50 ng/ml). Our findings were similar to other researchers reported that EGF was contributed to the promotion of oocyte maturation (Downs, 1989; Sanbuissho et al., 1991). The results of the present study revealed that the IVM supplemented with EGF (20 ng/ml) increased the oocyte maturation, fertilization, cleavage, morula and blastocyst production rates. These results were similar to other studies achieved by Singhal et al., (2009) and Mishra et al., (2010) which used EGF as supplements during IVM of buffalos' oocytes. Moreover, EGF supplementation to the IVM media doubled the blastocyst formation rate (13 to 27%) (Grazul- Bilska et al., 2003). In addition, our findings supported the results reported by Kandil et al. (2013) and Sadeesh et al. (2014) which mentioned that the optimum concentration of EGF added to the IVM media was 20 ng/ml concentration to maintain better

development of buffalo embryos. Other groups stated that oocytes maturation and embryo development rate were increased at concentration 10 ng/ml EGF in several animal species as cow (Lonergan et al., 1996 and Mtango et al., 2003) and buffalo (Kumar and Purohit, 2004 and Purohit et al., 2005). Moreover, Sirisathien et al. (2003); Thongkittidilok et al. (2015) and El-Naby et al. (2016) observed that oocyte competence and blastocyst developmental rate were improved at a concentration of 5 ng/ml EGF compared with the control group. While the present study recorded that the low concentrations as 10 ng/ml decrease the maturation and embryo development compared to 20 ng/ml.

In accordance to our study, Carpenter and Cohen, (1976) and Sirotkin et al., (2000) stated that higher concentrations (40 ng/ml) of EGF reduced the blastocyst development which is likely due downregulation of the EGF-induced receptor. The blastocyst development decreased at a higher concentration of EGF (50 ng/ml). Thus the presence of high concentration of EGF caused a significant down regulation of the EGF-induced receptors or acceleration of EGF receptors degradation (Beguinot et al., 1984). In another study using sheep, Ni et al. (2015) proved that in vitro embryo developmental rate was significantly higher at concentration of 50 ng/ml EGF. While, the suitable concentration for mouse embryo development was 1 ng/ml EGF (Merriman et al., 1998).

Conclusion

From the present study, it was concluded that addition of EGF at 20 ng/ml concentration in IVM media enhance the maturation of oocyte, fertilization and developed embryos. Addition of EGF to the maturation medium had an economic value owing to increase the percent of developed embryos compared to the control group.

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