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EFFECT OF INSULIN-LIKE GROWTH FACTOR-I AND EPIDERMAL GROWTH FACTOR ON IN VITRO EMBRYO PRODUCTION IN CATTLE

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ABSTRACT

The purpose of this study was to investigate the effect of supplementing in vitro maturation medium and culture medium with insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and a combination of them on the development of in vitro–matured and fertilized (IVM/IVF) bovine oocytes, as well as their developmental rate to cleavage and blastocyst stages. For this purpose, 464 bovine ovaries were collected from Beni Suef abattoir; oocytes were aspirated and used for in vitro maturation and subsequent embryo development. The current results revealed that supplementing maturation and culture media with a combination of IGF-I and EGF was the best treatment for improving rates of IVM, IVF, cleavage and blastocyst development. Addition of IGF-I or EGF into maturation medium had insignificant effect on IVM rate but significantly enhanced subsequent embryonic development as compared to control. Moreover, including IGF-I in culture medium significantly improved cleavage, morula and blastocyst rate as compared to EGF-group.

Key words: *Insulin-like growth factor-I, epidermal growth factor, in vitro embryo production, cattle*

INTRODUCTION

In vitro maturation of bovine oocytes involves removal of prophase I oocytes (together with surrounding cumulus cells) from antral follicles and their culture for 20 to 24 hrs. (Galli *et al.,* 2003). During this interval, oocytes extrude their first polar bodies and reach metaphase II (Gilchrist and Thompson, 2007). Supplementation of the maturation medium with selected serum, hormones and other active factors markedly enhanced quality of the matured oocyte. Maturation was better aided by the presence of proestrus or estrus cow because, the serum containing high LH concentrations (Brackett and Zuelke, 1993). Further refinement of conditions for in vitro maturation included addition of various growth factors (Brackett, 2001).

A range of polypeptide growth factor ligands and their receptors have been found to be expressed and produced in the reproductive tract or the preimplantation embryo, in particular EGF and IGF-I and II. Growth factors and cytokines have very important regulatory role during pregnancy (Block *et al.,* 2003 and Spencer *et al.,* 2008). Insulin and insulin like growth factors are major factors that regulate oocytes maturation by acting synergistically with FSH as autocrine and paracrine modulators of granulosa cells, thereby, promoting mitogenesis, steroidogenesis and protein synthesis (Spanos *et al.,* 2000 and Zicarelli and Gasparrini, 2004) leading to enhanced oocyte maturation, fertilization and embryo development to blastocyst stage (Pawshe *et al.,* 1998 and Purohit *et al.,* 2005). IGF-I promotes oocyte maturation and reduces apoptosis during early human preimplantation embryos (Kawamura *et al.,* 2005 and Neira *et al.,* 2007). Moreover, IGF-I and II are expressed and produced in ruminant embryos from the zygote stage to implantation in the ICM and in the trophoblast; they then continue to be expressed until at least Day 30 of gestation (Martal *et al.,* 1998). In this respect, the addition of IGF-I and II to the culture medium appears to be beneficial as they increase the number of embryos that develop to blastocyst stage in human, mouse, porcine, ovine, and bovine (Watson *et al.,* 1994; Spanos *et al.,* 2000; Glabowski *et al.,* 2005 and Spencer *et al.,* 2008).

Epidermal growth factor stimulates DNA synthesis in cumulus cells (Khamsi and Armstrong, 1997), pattern of protein neosynthesis (Lonergan *et al.,* 1996), production of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) by cumulus cells which stimulates oocyte maturation (Park *et al.,* 1999). Moreover, studies in mouse, rat, human, porcine, and bovine have demonstrated that EGF stimulates in vitro maturation (Reed *et al.,* 1993; Lonergan *et al.,* 1996; Lighten *et al.,* 1997 and Rieger *et al.,* 1998).

Therefore, the current study aimed to clarify the effect of IGF-I and/or EGF supplementation during in vitro maturation or culture on the maturation and fertilization of bovine oocytes as well as their developmental rate to cleavage and blastocyst.

MATERIALS and METHODS

All reagents for media preparation were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). To study the effect of growth factors on bovine in vitro embryo production, two separate experiments were done: First experiment: 243 cattle ovaries were used. During maturation, the oocytes were subjected to 4 treatments; Group 1 (Control: maturation medium "MM" alone), Group 2 (MM+ IGF-I; 100 ng/ml), Group 3 (MM+ EGF; 20 ng/ml) and Group 4 (MM+ IGF-I+ EGF).

Second experiment: 221 cattle ovaries were used and the same treatments of the first experiment were implemented during culture of embryos.

1. Ovaries collection and oocyte recovery
Cattle ovaries, in warm sterile saline supplemented with 50 µg/ml gentamycin in insulated container were transported to the laboratory within 1 hr of slaughter. Upon arrival, the ovaries were washed repeatedly in normal saline, trimmed free of extraneous tissue and rinsed in normal saline. The cumulus-oocytes complexes (COCs) were recovered from follicles by the slicing method and subsequently washed three times in tissue culture medium 199 (TCM-199) supplemented with 50 µg / ml gentamycin sulfate and 5 µl / ml L-glutamine (washing medium). The COCs were assessed morphologically, using a low-power (X40) stereomicroscope, and only those having evenly granulated cytoplasm and completely surrounded by cumulus cells were selected for in vitro maturation (Pawshe *et al.,* 1994).

2. In vitro maturation
COCs were washed twice in TCM-199 supplemented with 10 % FCS, 50 µg / ml gentamycin sulfate and 5 µl / ml L-glutamine (Nedambale *et al.,* 2006) then transferred to 50 µl TCM-199 maturation medium supplemented with 10 % FCS, 50 µg / ml gentamycin sulfate and 5 µl / ml L-glutamine, 0.2 i.u FSH, 2.0 i.u HCG and 1.0 µg E_2 / ml (Choi *et al.,* 2001).

The oocyte - containing droplet (10 cells) was covered with 4 ml sterile mineral oil to prevent evaporation then incubated for maturation in CO_2 incubator for 24 hrs (Nedambale *et al.,* 2006) after which they were examined under stereomicroscope (X 100). The assessment of maturation was done by the degree of expansion of cumulus cell mass and extrusion of first polar body into the perivitelline space.

3. In vitro fertilization (IVF) of oocytes
Motile spermatozoa were selected using swim-up technique in Sperm - Tyrod's Albumin Lactate Pyruvate medium (S- TALP) supplied with gentamycin sulfate (50 µg / ml) and BSA (Sigma-Aldrich, USA) fraction-V "essential fatty acid - free" (6 mg / ml) and heparin sodium salt (200 i.u. / ml) for sperm capacitation (Parrish *et al.,* 1986). For this purpose, two straws of frozen bull semen received from Artificial insemination center, Beni Suef, Egypt were used. The selected bovine sperm was resuspended in 1.0 ml of Fertilization Tyrod's, Albumin, Lactate, Pyruvate medium (F-TALP) supplied with 10 µg / ml penicillamine, 50 µg / ml gentamycin sulfate and 6 mg / ml BSA (Younis *et al.,* 1989). Sperm concentration was measured by hemocytometer and a sufficient medium was added to yield the final concentration of 1 X 10^6 sperm / ml.

Following maturation, good and excellent mature COCs were washed three times by F-TALP medium then transferred to 50 µl droplets of the same medium (5 oocytes / droplet). The oocytes were covered with warm sterile mineral oil then incubated in CO_2 incubator for an hour after which the oocytes were inseminated with sperm suspension (2 µl / droplet).

4. In vitro culture (IVC) of fertilized oocytes
Twenty four hours following fertilization, the inseminated oocytes were washed 3 times using hepes – TCM 199 " H-TCM " supplemented with 10 % FCS, 50 µg / ml gentamycin sulfate and 5 µl / ml L-glutamine (culture medium) then transferred to droplets of the same medium (5 oocytes / 100 µl) and incubated in the CO_2 incubator with change of the culture medium every 48 hours for 7 successive days (Hammam *et al.,* 1997). The culture media was changed with freshly prepared embryo culture media every 48 hrs and observation was made for cleavage and subsequent development of embryos.

5. Statistical analysis
The results obtained for in vitro maturation, fertilization and embryonic development of oocytes were analyzed using SAS user guide (SAS, 2004) to determine ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

1. Effect of IGF-I and EGF in maturation medium on in vitro embryo production:
The current results revealed that addition of IGF-I + EGF to IVM medium significantly increased IVM rate (94.3 ± 0.30%) as compared to control, IGF-I and EGF groups (90.5 ± 2.10, 91.3 ± 0.64 and 91.9 ± 0.85, respectively). However, there was no significant increase in the rate of IVM on adding IGF I or EGF. In regards to the effect of IGF-I and EGF on fertilization rate, it was found that addition of IGF-I

(69.70±0.52%) or EGF (69.50±0.60%) alone or in combination (73.04±0.94%) significantly enhanced IVF rate than the control group (63.90±0.87%). Similarly, including the maturation medium with IGF-I, or EGF alone or in combination resulted in significantly higher cleavage, morula and blastocyst % compared to control group. Moreover, Oocytes matured in the presence of IGF-I + EGF had higher rates of cleavage, morula and blastocyst formation than those in IGF-I or EGF groups (Table 1).

Addition of IGF-I alone or in combination with EGF, into cleavage medium, significantly increased cleavage, morula and blastocyst production rates as compared with control and EGF groups (Table 2).

Table 1: Effect of insulin like growth factor and epidermal growth factor in maturation medium on in vitro embryo production (Mean±SE)

Treatment	*Maturation (%)*	*Fertilization (%)*	*Cleavage (%)*	*Morula (%)*	*Blastocyst (%)*
Control	90.5±2.10^{a}	63.90±0.87^{a}	47.20±0.70^{a}	32.30±0.54^{a}	13.70±0.35^{a}
IGF-I	91.3±0.64^{ab}	69.70±0.52^{b}	50.10±0.93^{b}	35.30±0.72^{b}	17.20±0.64^{b}
EGF	91.9±0.85^{ab}	69.50±0.60^{b}	50.60±0.84^{b}	35.70±0.59^{b}	16.80±0.64^{b}
IGF+EGF	94.3±0.30^{b}	73.04±0.94^{c}	54.20±0.63^{c}	37.60±0.52^{c}	20.60±0.86^{c}

Within the same column values with different superscript letters (a, b and c) were statistically different at (P≤0.05).
SE: standard error.

Table 2: Effect of insulin like growth factor and epidermal growth factor in culture medium on in vitro embryo production (Mean±SE)

Treatment	*Fertilization (%)*	*Cleavage (%)*	*Morula (%)*	*Blastocyst (%)*
Control	66.90±1.52	48.40±0.88^{a}	32.30±0.72^{a}	15.40±0.46^{a}
IGF-I	64.40±1.32	54.70±0.52^{b}	37.40±1.14^{b}	18.60±0.79^{b}
EGF	64.90±1.83	48.30±1.30^{a}	33.30±0.88^{a}	14.90±0.68^{a}
IGF+EGF	66.50±1.50	54.80±0.79^{b}	37.70±1.31^{b}	19.80±0.68^{b}

Within the same column values with different superscript letters (a, b and c) were statistically different at (P≤0.05).
SE: standard error.

DISCUSSION

The purpose of this study was to examine the effect of growth factors on early embryonic development. Throughout the study, two types of growth factors; IGF-I and EGF were tested on the basis of their known pleiotropic effects on embryonic development (Eckert and Niemann, 1998; Martal *et al.,* 1998 and Chaouat *et al.,* 2003). The concentrations of growth factors used were according to previous reports suggested that insulin like growth factor-1 (IGF-1) at 100 ng/ml (Pawshe *et al.,* 1998) and (EGF) 20 ng/ml (Gupta *et al.,* 2002) are the respective optimum concentrations for oocyte maturation and subsequent development.

Results of the current study showed that IGF-I or EGF added to maturation medium had no significant effect on IVM rate as compared to control while their combination significantly increased in vitro maturation of bovine oocytes. Moreover, addition of IGF-I and / or EGF to the maturation medium significantly enhanced in vitro fertilization, cleavage, morula and blastocyst rates. These results come in accordance with those of Oyamada and Fukui (2004) who used EGF and cysteamine together during IVM and found no positive effect on nuclear maturation but improved cleavage rate and developmental competency of oocyte. In addition, results obtained by Sakaguchi *et al.* (2000) indicated that the progression of meiosis in bovine oocytes with cumulus cells is accelerated by exposure to combination of IGF-I and EGF in serum-free maturation medium. These results, also, coincide with those obtained by Purohit *et al.* (2005) and Wani *et al.* (2012) who recorded significant increase in

cleavage rate and subsequent embryo development with EGF supplementation in bovine and ovine, respectively. Harper and Brackett (1993) reported that addition of growth factors; EGF and IGF-I had beneficial effect on blastocyst production rate in a number of species. Such beneficial effect may be due to the fact that EGF has been shown to stimulate nuclear and cytoplasmic maturation in a wide variety of species, including mouse, pig, cattle, deer, goat and sheep (Lonergan *et al.,* 1996; Abeydeera *et al.,* 2000; Guler *et al.,* 2000 and Comizzoli *et al.,* 2001) and subsequently enhance blastocyst yields (Park *et al.,* 2004). Insulin like growth factors are major factors that regulate oocytes maturation by acting synergistically with FSH as autocrine and paracrine modulators of granulosa cells, thereby, promoting mitogenesis, steroidogenesis and protein synthesis (Spanos *et al.,* 2000 and Zicarelli and Gasparrini, 2004) leading to enhanced oocyte maturation, fertilization and embryo development to blastocyst stage (Pawshe *et al.,* 1998 and Purohit *et al.,* 2005). Furthermore, IGF-I reduces apoptosis during early human preimplantation embryos (Kawamura *et al.,* 2005 and Neira *et al.,* 2007).

In the second experiment, addition of IGF-I alone or in combination with EGF, into cleavage medium, significantly increased cleavage, morula and blastocyst production rates as compared with control and EGF groups. Neira *et al.* (2007) reported that addition of 50 ng IGF-I to in vitro culture medium of bovine embryos significantly increased the blastocyst yields after 8 days of embryo culture. On the contrary, media containing EGF did not support embryonic development from 8-cell stage and thereafter, suggesting a possibility of EGF-causing atresia (Monniaux *et al.,* 1997). It is interesting to note that IGF-I and EGF also stimulate embryo development by increasing RNA and DNA synthesis at time of compaction, which is followed by an increase in blastocyst yield in vitro (Brison and Schultz 1997 and Young *et al.,* 2001). IGF-I also stimulates the incorporation of proteins by endocytosis and limits their degradation (Brison and Schultz, 1997). IGF-I increases total cell number in blastocysts exposed to stressors which increases the percentage of blastomeres that undergo apoptosis (Jousan and Hansen, 2007 and Block *et al.,* 2008). IGF-I and EGF have a positive effect on preimplantation of embryo development under detrimental culture conditions of oxidative stress (Kurzava *et al.,* 2004 and Yang *et al.,* 2005).

CONCLUSION

In conclusion, the supplementation of IGF-1 + EGF together with basic maturation medium and culture medium exerted a positive influence on the progression of oocyte maturation and improved IVF and consequently could be utilized for efficient in vitro embryo production in cattle.

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تأثير عامل النمو شبية الأنسولين وعامل نمو البشرة المضاف الى بيئة النضوج والإخصاب المعملى على معدل إنتاج الإجنة فى الأبقار

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استهدفت هذه الدراسة معرفة تأثيركل من عامل النمو شبية الأنسولين وعامل نمو البشرة المضاف الى بيئة النضوج والإخصاب المعملى على معدل النضوج والإخصاب للبويصات البقرية بالإضافة الى معدل الإنقسامات ونمو الإجنة. استخدم فى هذة الدراسة عدد (٤٦٤) من مبايض الابقارمن مجزر بنى سويف. تم سحب البويضات موضع الدراسة بطريقة التقطيع العرضى والطولى للمبايض. اسفرت النتائج فى هذه الدراسة ان عامل النمو شبية الأنسولين وعامل نمو البشرة من احسن الإضافات الى بيئة النضوج والإخصاب المعملى والتى حققت معدل زيادة فى نضوج البويضات ومعدل الإخصاب والإنقسامات عند اضافتهما معا. بينما اسفرت النتائج انة لا يوجد تأثير معنوى عند اضافة كل منهما على حدة على معدل نضوج البويضات بينما كان التأثير واضحا على معدل الإنقسامات ونمو الإجنة لكل منهما على حدة.