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Effect of growth factor on *in-vitro* maturation of porcine oocyte

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ABSTRACT

The present study was aimed to evaluate the beneficial effect of different growth factors on *in-vitro* maturation of porcine oocytes. Ovaries were collected from a local abattoir immediately after slaughter of the animals and transported to the laboratory. A total of 618 type A and type B oocytes were cultured in TCM-199 containing additives with PMSG and hCG for the first 22 hrs and without hormones for subsequent 22 hrs of incubation at 39°C under 5 per cent CO_2 level and 90-95 per cent humidity. The effects of supplementation of different growth factors *viz.,* EGF, IGF-I and EGF + IGF-I in the medium were studied. The rate of oocytes with cumulus cells expansion was significantly higher ($P<0.01$) when growth factors were added as compared to control but it did not differ significantly between growth factors. The rate of nuclear maturation of oocytes was significantly higher ($P<0.01$) as compare to control for EGF and EGF + IGF-I but not for IGF-I. There was no significant difference in the rate of oocytes with nuclear maturation between the growth factors studied. It can be concluded from the present study that addition of EGF, IGF-I or EGF + IGF and additives along with hormones (PMSG and hCG for first 20-22 hrs) in TCM-199 Medium gives optimum *in-vitro* maturation rates in porcine oocytes.

Key words: Growth factor, IVM, Porcine oocyte, TCM-199.

INTRODUCTION

Pig rearing is one of the most important occupation of rural society of the North Eastern region of the country specially the tribal masses. Pig contributes 40 per cent of the world's meat production and is the most important meat source globally (Dang *et al*., 2010). However, there is an impending threat to the genetic diversity of this important food animal, with 151 breeds of pigs already classed as extinct and 132 more breeds at risk (FAO, 2000). Pig is now becoming increasingly important in the field of biomedical research and interest has grown in use of transgenic pigs by nuclear transfer or cloning technique. The development of efficient *in-vitro* techniques would allow the production of large numbers of mature oocytes and embryos in shorter time and with lower costs. Furthermore, IVP of embryo enables the production of large number of offspring from live or slaughtered animals.

Changes in the composition of follicular fluid during follicular growth affect various metabolic events in growing oocytes. Perusal of available literature showed beneficial effects of growth factors (Abeydeera *et al*., 2000) on *in-vitro* oocyte maturation. However, there is diversity of opinion about the best growth factor for *in-vitro* maturation of oocytes. Despite recent developments in this field, there are problems such as a high incidence of polyspermy and low rates of blastocyst formation, which may be partly due to incomplete maturation and poor quality of oocytes during IVM of porcine oocyte (Krisher 2004). However, a few studies have been reported to increase monospermic fertilization and subsequent embryo development from *in-vitro* matured porcine oocytes with diversed results. Perusal of available literature revealed no report from India on IVM/IVF of porcine oocyte. Hence, the present study was undertaken to study the effect of growth factor on *in-vitro* porcine oocyte maturation.

MATERIALS AND METHODS

Porcine ovaries were collected from local abattoirs of Shillong immediately after slaughter of the animal and transported to the laboratory within 2-3hours in a thermos flask containing NSS (0.9%) with antibiotic (Gentamicin) maintaining 37-39ºC. In the laboratory extraneous tissues adhering to the ovaries were removed with scissors and washed 3-4 times with Phosphate Buffer Saline (PBS) containing antibiotic to remove excessive debris. Aspiration of oocytes from follicles was done with 18 gauge needle and 10 ml syringe. Presence of oocytes in the petri dish containing aspiration medium was searched under a steriozoom microscope. After recovery, the oocytes were graded on the basis of number of cumulus cell layer adhered to the zonapellucida as Type A, B, C and D. Oocytes were washed 3-4 times in washing medium and finally two times in maturation medium. Oocytes were matured in four

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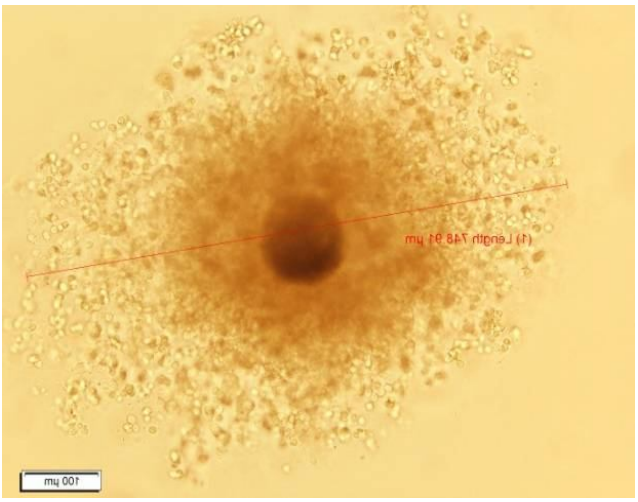

Fig 1: cumulus cells expansion after ivm

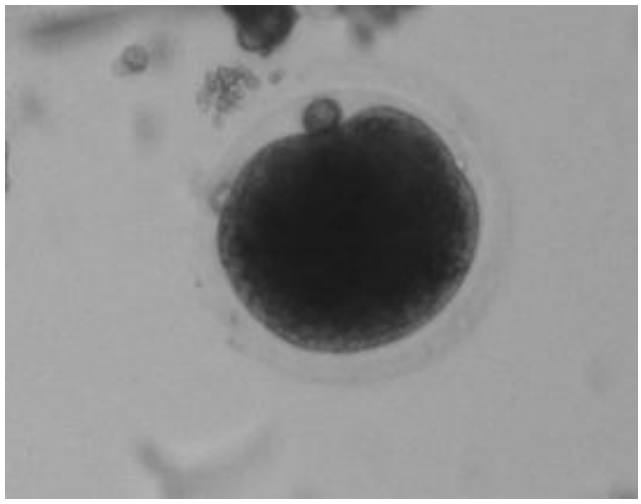

Fig 2: First polar body extrusion

different IVM media added with different additives in the base medium TCM-199. The control medium contained 7.5ml TCM-199, 1ml Fetal calf serum, 1 ml Porcine follicular fluid, 300µl Gentamicin, 100 µl sodium pyruvate (0.1%) and 100 µl Non essential amino acids while treatment medium included addition of 10ng/ml EGF or 50ng/ml IGF-I or combination of both EGF and IGF-I at the same concentration to the control medium. Each medium was divided into two equal fractions (Fraction A and Fraction B) containing 5ml in each. Fraction A was added with hormones (10 IU hCG and 10 IU PMSG/ ml of medium).With the help of a micro pipette a 50 µl droplet of fraction A maturation medium (as IVM droplet) was placed gently on a 35mm petri dish. Oocytes were transferred to the droplet @12-15 no per droplet and covered with warm (38-39ºC) mineral oil. The petridish was covered and placed inside a 95mm petridish and incubated in CO_2 incubator maintaining 5 per cent CO_2 and 95 per cent relative humidity for 22-24 hours. After 22-24 hrs oocytes were transferred to fraction B part of maturation media (without hormones) and incubated for next 22-24 hrs in the same condition. Oocyte maturation was assessed based on the cumulus cells expansion (Fig.1) and extrusion of first polar body (Fig.2). Oocytes were nuded with the help of gentle pipetting in hyaluronidase drop for 5 minutes. The oocytes were fixed with the help of acetic alcohol (acetic acid 1 part and ethyl alcohol 3 parts) and stained with 1% aceto-orcin stain (Martin, 2000).

RESULTS AND DISCUSSION

In the present study the rates of cumulus cells expansion of oocytes matured in medium with different growth factors ranged from 78.40 to 82.05 per cent (Table 1). The expansion of cumulus cells of oocytes was found to be the highest in medium with EGF and IGF-I (82.05%) followed by EGF (81.53%), IGF-I (78.40%) and control (55.35%). In the present study the percentage of cumulus cells expansion of oocytes was significantly higher ($P< 0.01$) in medium with all the growth factors in comparison with the control medium but there was no significant difference between the growth factors supplemented in the medium (Table 2).

Sofi *et al.* (2011) and Kumar and Purohit (2004) reported similar results (82.90% and 81.4%) in medium with EGF in ovine and buffalo oocytes respectively. Bastan *et al.* (2010) recorded 95.10 per cent cumulus cells expansion rate in bovine oocytes cultured in medium supplemented with 10ng/ml EGF which was higher than the present finding. Prochazka *et al.* (2011) reported lower cumulus cells expansion rate (56.00%) in pig oocytes when supplemented the medium with EGF like peptide. Lorenzo *et al.* (1995) reported lower cumulus cells expansion rate in medium with EGF (62.6%), IGF-I (40.6%) and EGF+IGF-I (74.2%) in bovine oocytes when compared with the present study.

The rates of nuclear maturation of oocytes matured in medium with different growth factors varied from 54.83

Table 1: Effect of addition of growth factors in tcm-199 medium containing additives on in-vitro expansion of cumulus cells of oocytes

Growth factor inmedium	Number of oocytes incubated	Number of oocytes with cumulus cellsexpansion	Percentage	Chi-square value
TCM-199+additives +EGF	195	159	81.53	17.96**
TCM-199+additives +IGF-I	176	138	78.40	
TCM-199+additives +EGF+ IGF-I	39	32	82.05	
TCM-199+additives (Control)	56	31	55.35	

**$P < 0.01$

Table 2: Effect of addition of growth factors in tcm-199 medium containing additives on *in-vitro* expansion of cumullus cells of oocytes showing independent chi square values

	TCM-199+additives +EGF	TCM-199+additives +IGF-I	TCM-199+additives +EGF+IGF-I	TCM-199 +additives(Control)
TCM-199+additives+EGF	-	0.57 ^{NS}	0.005 ^{NS}	16.21**
TCM-199+additives+IGF-I	-	-	0.26^{NS}	11.41**
TCM-199+additives+EGF+IGF-I	-	-	-	7.33**
TCM-199+additives(Control)	-	-	-	-

**P<0.01,^{NS} Non-significant

to 68.18 per cent (Table 3). In the present study nuclear maturation was found to be the highest in medium with EGF + IGF-I (68.18%) followed by EGF (64.58%), IGF-I (54.83%) and control (34.14%). The rate of nuclear maturation was found to be significantly higher (P<0.01) in medium containing EGF and EGF + IGF-I from that of control medium but there was no significant difference between the medium containing IGF-I and control medium (Table 4). No significant difference was observed in nuclear maturation rate of oocyte between medium with different growth factors.

Similar results were obtained by Marques *et al*. (2007) in medium with EGF (65.00 %) in pig oocytes. Abeydeera *et al*. (2000) and Kishida *et al.* (2004) reported higher maturation rates of 84.8 and 77.4 per cent respectively in medium with EGF in pig oocytes. Oberlender *et al*. (2013) reported higher level (81.8%) of nuclear maturation of oocytes in media with IGF-I in pig than the present study. In another study Kumar and Purohit (2004) reported higher maturation rates of 76.55, 81.34 and 82.93 per cent in medium with EGF, IGF-I and EGF+IGF-I respectively in buffalo oocytes. The variations in the incidence of cumulus cells expansion and nuclear maturation reported by various workers as compared with the present investigation might be due to the variations in species, composition of media, additives, concentration of growth factors used, and period and temperature of incubation of oocytes.

Reed *et al*. (1993) reported that the addition of EGF to IVM medium supplemented with gonadotropins significantly stimulated the nuclear maturation of pig oocytes. Coskun and Lin (1993) and Lorenzo *et al*. (1994) observed that exogenous EGF induced oocyte maturation by generating a positive signal in the cumulus cells in pig and cattle respectively. Many workers reported that EGF contributed to the promotion of oocytes maturation (Downs, 1989), germinal vesicle breakdown and polar body formation (Das *et al.,* 1991). Nagar and Purohit (2005) stated that goat oocytes matured *in-vitro* in the presence of EGF recorded a greater cumulus cells expansion and higher maturation rate than control oocytes. However, Wang and Niwa (1995) found that addition of EGF during IVM did not stimulate further cumulus expansion in porcine cumulus oocyte complexes in the presence of gonadotropins and serum. Xia *et al.* (1994) found that IGF-I had a beneficial effect on *in-vitro* oocytes maturation at physiological doses. Addition of IGF-I to culture media *in-vitro* was found to promote maturation of oocytes (Lorenzo *et al*., 1994).

Fenk *et al*. (1987) found that the presence of the receptors for the growth factors in the granulosa cells or in oocytes which might suggest the involvement of these factors

Table 3: Effect of addition of growth factors in tcm-199 medium containing additives on *in-vitro* nuclear maturation of oocytes

Growth factor in medium	Number of oocytes incubated	Number of oocytes matured	percentage	Chi-square value
TCM-199 +additives +EGF	48	31	64.58	10.46*
TCM-199 + additives +IGF-I	31	17	54.83	
TCM-199 + additives +EGF+ IGF-I	22	15	68.18	
TCM-199 + additives (Control)	41	14	34.14	

*P < 0.05

Table 4: Effect of addition of growth factors in tcm-199 medium containing additives on *in-vitro* nuclear maturation of oocytes showing independent chi square values

	TCM-199+additives +EGF	TCM-199+additives +IGF-I	TCM-199+additives +EGF+IGF-I	TCM-199 +additives (Control)
TCM-199+additives+EGF	-	0.75 ^{NS}	0.09 ^{NS}	8.19**
TCM-199+additives+IGF-I	-	-	0.96^{NS}	3.08 ^{NS}
TCM-199+additives+EGF+IGF-I	-	-	-	6.68**
TCM-199+additives(Control)	-	-	-	-

**P<0.01, ^{NS} Non-significant

in the maturation process. Singh *et al*. (1995) reported the presence of mRNA for EGF and its receptor (EGF-R) in the oocyte, cumulus and granulosa cells indicating synthesis of EGF by these tissues. This was further supported by the localization of EGF peptide in the oocyte, cumulus and granulosa cells of all stages of follicles. The higher maturation rate recorded for the EGF-containing medium might be due to the probable expression of EGF receptors in the cumulus cells and EGF triggering the signalling via the mitogen activated protein kinase (MAPK) pathway (Gall *et al*., 2004, 2005). Shimada *et al*. (2006) reported that the cumulus cells expansion was triggered by factors like EGF, since it stimulated the expression of several genes crucial for expansion such as prostaglandin-endoperoxide synthase 2 (PTGS2) also known as cyclooxygenase 2 (COX2), hyaluronan synthase 2 (HAS2), tumor necrosis factor alpha-induced protein 6 (TSG6) and pentraxin 3 (PTX3). *In-vitro* stimulation by EGF improved the cAMP production by the cumulus-oocyte complexes which induced the breakdown of the germinal vesicle (Downs *et al*., 1991). EGF could significantly stimulate the synthesis of GSH in oocytes which could reduce di-sulphide bonds and protected cells against oxidative damage (Chance *et al*., 1979).

In the present study *in-vitro* maturation of oocytes was carried out in presence of porcine follicular fluid and gonadotropin. Porcine follicular fluid contained many unknown factors (Hsu *et al.,*1987) that might have acted synergistically with EGF to stimulate cumulus cells expansion. Ding and Foxcroft (1994) observed that in pigs EGF could stimulate nuclear maturation and could interact with gonadotropins to enhance cytoplasmic maturation. However, Merlo *et al*. (2005) reported that EGF did not significantly enhance the nuclear maturation rate, but enhanced the cytoplasmic maturation. In the present work the percentage of oocytes with expanded cumulus cells was the lowest in the medium supplemented with IGF-I. This could probably either because IGF did not act via cumulus cells or it interfered with the production of an expansion factor produced by the oocyte. The rate of *in-vitro* nuclear maturation of oocytes in TCM-199 + additives + IGF-I did not vary significantly from that in control which revealed that supplementation of IGF-I was not efficacious. Grupen *et al*. (1997) also reported that the addition of IGF-I had no effect on meiotic maturation of porcine oocytes *in-vitro.* EGF being a mitogenic factor had the ability to stimulate the proliferation of ovarian granulosa cells (May *et al.,*1987).The highest rate of cumulus cells expansion and nuclear maturation of oocytes with supplementation of EGF + IGF-I could be attributed to their synergistic effect since EGF and IGF-I in combination had been shown to act synergistically and to accelerate the cumulus cells expansion and the progression of meiosis (Lorenzo *et al.,* 1994).

It can be concluded from the present study that addition of EGF, IGF-I or EGF + IGF and additives along with hormones (PMSG and hCG for first 20-22 hrs) in TCM-199 Medium gives optimum *in-vitro* maturation rates in porcine oocytes.

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