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Effect of hormones, follicular fluid, serum and media on *in vitro* maturation of porcine oocyte

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ABSTRACT

The present study was conducted to evaluate the effect of hormones, follicular fluid, serum and media on *in vitro* maturation (IVM) of porcine oocyte. The procedures of IVM could be used for basic research purposes or commercial utilization. The IVM of oocyte is a critical step in *in-vitro* embryo production and it needs to be carried out in a precise manner under optimal conditions for subsequent fertilization and embryo development. For this purpose ovaries were collected from slaughtered pigs from an abattoir. Ovaries (1,232) were aspirated in 52 trials for IVM and fertilization for this study. Dulbecco Modified Eagle's medium (DMEM) or tissue culture medium (TCM-199) supplemented with gentamycin (50µl/ml), sodium pyruvate (100 mmol) and 10% serum, viz. estrus sow serum (ESS) or fetal calf serum (FCS) with or without porcine follicular fluid (PFF) and hormones such as pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) were used. The maturation was assessed by loosening of cumulus cells, enlargement of peri-viteline space or extrusion of first polar body and examination of metaphase II by aceto-orcein stain. The results revealed that DMEM with ESS yielded 53% while TCM+ESS yielded 45% maturation rates. The replacement of ESS with FCS resulted in 48.50 and 35.33% maturation in DMEM and TCM-199 medium, respectively. The addition of PFF in DMEM+ESS improved the maturation rate and was found better (74.55%) than TCM-199+ESS (65.35%). However, the replacement of ESS by FCS in either DMEM orTCM-199+PFF did not differ significantly. It can be concluded from the present study that use of DMEM+ESS+PFF along with hormones PMSG + luteinizing hormone (LH) for first 20– 24 h followed by hormone free medium for next 20– 22 h i.e. 42–44 h gave optimum (81.5%) *in-vitro* maturation rates in pigs. DMEM with ESS and PFF with hormone (PMSG/HCG) yielded highest maturation (81.50%) rate than TCM-199 with ESS, PFF and hormone (PMSG/HCG) which showed maturation rate of 76.59%.

Key words: Follicular fluid, Hormone, *In-vitro* maturation, Oocyte, Porcine, Serum

The ovarian follicle, the basic structural and functional unit of the mammalian ovary, provides the micro-environment necessary for oocyte growth and maturation (Zeleznik 1993). Developing a follicular culture system will result in fertilizable oocytes that would be advantageous not only for understanding folliculogenesis but also for the preservation and long-term storage of female germ cells. Transplantation of cryopreserved primordial follicles to sterilized animals can restore fertility (Carroll and Gosden 1993).

Oocyte maturation is one of the most important stages for *in vitro* production of embryos. Several media support *in vitro* nuclear maturation of pig oocytes but not cytoplasmic maturation. Problems in cytoplasmic maturation interfere with the formation of the pronuclei after penetration of the sperm, despite normal germinal vesicle (GV) breakdown and extrusion of the first polar body (Abeydeera 2002). Yoshida *et al*. (1992) concluded that porcine follicular fluid (PFF) contains substances that improve the expansion of the cells of the *cumulus oophorus*, nuclear maturation and normal fertilization. PFF contains high concentrations of superoxide dismutase and it also plays an important role in the protection of oocytes against oxidative stress (Tatemoto *et al*. 2004). Addition of blood serum as a supplement to the culture medium provides a superior environment for oocyte maturation (Leibfried *et al.* 1986). Use of follicular fluid to replace serum to increase the efficiency of maturation was proposed, since the follicular fluid is composed of follicular secretions, which *in vivo* supports oocyte maturation (Naito *et al.* 1989, Rath *et al.* 1995), while no beneficial influence of the follicular fluid in male pronucleus (MPN) formation (Rahman *et al*.

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2008). Maturation media supplementation plays a vital role on development potential of oocytes in IVP procedure. Tissue culture medium-199 (TCM-199) is used as a basic medium for IVM of goat oocytes but to establish well-defined medium addition of hormones, vitamins along with different protein supplements are also being used. The follicular fluid (FF) is a microenvironment that contains molecules involved in oocyte maturation, ovulation and fertilization. Microenvironment of developing follicles is critical to the acquisition of oocyte developmental competence, which is influenced by several factors including follicle size and season. Presence of hormones also affects oocyte maturation *in vitro*, especially of gonadotropins (Mattioli *et al.* 1988, Liu *et al.* 1997). Although the mechanism of action of LH and FSH during maturation is not very well understood, the addition of these hormones during maturation may provide better results. Therefore, the present study was planned to study the effect of media, follicular fluid, serum and hormones on *in-vitro* maturation of porcine oocyte collected from pigs reared under agro hill ecosystem of Meghalaya.

MATERIALS AND METHODS

Collection of ovaries: Porcine ovaries (1,232) were collected from local abattoir (Shillong) in normal saline (NS) at 37°C supplemented with 50µg/ml gentamicin sulphate and transported to laboratory within 1–2 h of slaughter. In the laboratory, tissue debris present on the ovaries were trimmed and washed with phosphate buffer saline (PBS) solution.

Collection of oocytes: The oocytes were collected by aspiration method using 10 ml glass syringe with 20 G needle containing Dulbecco phosphate buffered saline (DPBS) + 0.3% bovine serum albumen (BSA) + penicillin 100 IU/ml and streptomycin 100 µg/ml). Follicular contents were placed in sterile 15 ml centrifuge tubes and allowed to settle down at 37ºC in biochemical oxygen demand (BOD) incubator. The follicular content from centrifuge tubes were poured into sterile 90 mm disposable petri-dishes with grids and screened under stereo-zoom microscope. The oocytes were picked up and washed 3 times in maturation media.

In- vitro *maturation (IVM) of oocytes:* Trials (52) were conducted for *IVM* and *in-vitro* fertilization (IVF) studies using either DMEM or TCM-199 maturation medium supplemented with gentamycin 50µl/ml, sodium pyruvate (100 mmol) and10% serum, viz. estrus sow serum (ESS) or fetal calf serum (FCS) with or without porcine follicular fluid (PFF) and hormones pregnant mare serum gonadotropin (PMSG)/luteinizing hormone (LH) for first 24 h followed by hormone free medium for another 20–22 h under 5% CO_2, 95% humidity in CO_2 incubator (Fig.1). The maturation was assessed by observing the loosening of cumulus cells, enlargement of periviteline space and /or extrusion of first polar body and examination of metaphase II by aceto-orcein stain (Fig. 2).

Statistical analysis: The collected data were subjected to statistical analysis using the Student t-test or one-way ANOVA techniques by standard methods (Snedecor and Cochran 1994). Statistical analysis system (SAS) with 9.2 version software was used for the analysis of data.

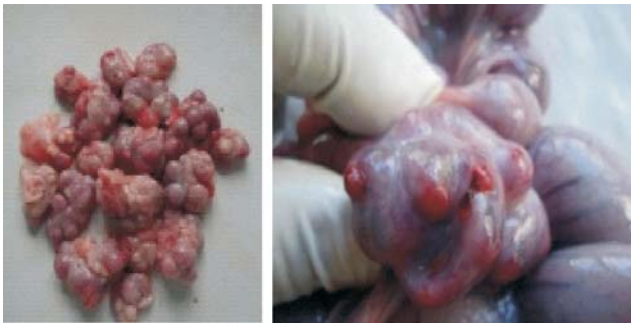

Fig. 1. Porcine ovaries collected from local abattoir of Meghalaya.

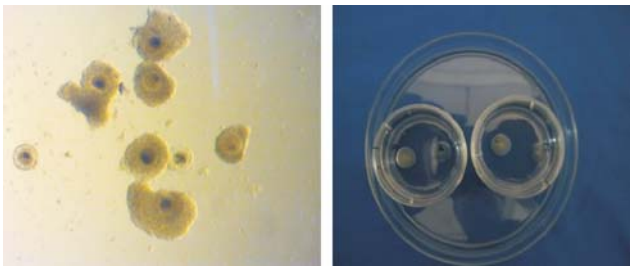

Fig. 2. *In –vitro* maturation of porcine oocytes.

RESULTS AND DISCUSSION

The results of *in-vitro* maturation with DMEM and TCM-199 medium (Tables 1, 2) revealed that DMEM with ESS yielded 53% while TCM+FCS yielded 45% maturation rates. The replacement of ESS with FCS resulted in 48.50 and 35.33% maturation in DMEM and TCM-199 medium, respectively. The addition of PFF in DMEM + ESS

Table 1. *In-vitro* maturation with serum in DMEM medium

Maturation media	Number of oocytes	Number of matured oocytes	Maturation (%)
DMEM+ESS	450	239	53
DMEM+FCS	450	219	48.5
DMEM+ESS+PFF	652	487	74.55
DMEM+ESS+PFF+ PMSG+HCG	550	449	81.50
Total	2102	1394	66.31

Table 2. *In-vitro* maturation with serum in TCM-199

Maturation media	Number of oocytes	Number of matured oocytes	Maturation (%)
TCM-199+ESS	450	203	45
TCM-199+FCS	450	159	35.33
TCM-199+ESS+PFF	788	515	65.35
TCM-199+ESS+PFF PMSG+hCG	650	498	76.59
Total	2318	1375	59.31

improved the maturation rate and was found better (74.55%) than TCM-199 + ESS (65.35%). However, the replacement of ESS by FCS in either DMEM or TCM-199+PFF did not differ significantly.

The present findings are in agreement with Naito *et al.* (1989) and Yoshida *et al.* (1992) who demonstrated that FF induced oocyte maturation *in vitro* improved the rate of male and female pronuclei formation and subsequent developmental capacity in pigs. Furthermore, both complete media porcine (Hirao *et al.* 1994) and simple medium porcine (Wu *et al.* 2001) were used for the culture of preantral follicles. Larocca *et al.* (1993) by comparing the effects of FF and oestrous cow serum reported that the presence of FF in culture medium during IVM-IVF of bovine oocytes increased the fertilization rate and percentage of morulae / blastocysts. Kim *et al.* (1996) also observed the beneficial effects of the addition of follicular fluid to the maturation medium on the maturation and developmental ability of bovine oocytes. Bovine and porcine oocytes matured and fertilized *in vitro* were found capable of developing to the blastocyst stage in serum-free medium (Pinyopumminytr and Bavister 1991, Abeydeera 2002). The ESS, PFF and hormones were to be the most appropriate supplements in our finding, which is in agreement with the findings of Abeydeera *et al.*(1998) and Hirao *et al.* (1994). One possibility is that gonadotropins stimulate some substance on the cumulus and/or granulosa cells which would act in the oocyte. This possibility is supported by the results of studies on localization of LH receptors in rat ovaries (Burovsky *et al.* 1993). The concentrations of LH and FSH used in this experiment seem to be unnecessary, since much lower concentrations can be used in the maturation medium without losing the effectiveness in improving maturation (Liu *et al.*1997). Dode and Graves (2001) reported that neither supplement nor hormone alone are capable of supporting pig oocyte IVM and cytoplasmic maturation rates can be increased by supplementing with follicular fluid. Ducolomb *et al*. (2013) identified the proteins and cryptides in porcine follicular fluid (PFF) that can stimulate porcine oocyte *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) when added to culture medium. Bertoldo *et al*. (2013), reported that there is a distinct shift in follicular glucose metabolism as follicles increase in diameter and suggested that follicular cells may be more vulnerable to oxidative stress during the summer. Follicular fluid is commo7nly added to porcine IVP of pig embryos media because of its capacity to increase successful maturation rate, promote male pronucleus formation, and increase the rate of monospermic fertilization. The benefits from FF may be augmented by growth factors, amino acids, and hormones, among other substances that promote oocyte maturation and fertilization (Algriany *et al*. 2004). Our finding also supported the above observation.

From the present study, it can be concluded that use of DMEM+ESS+PFF along with hormones (PMSG+hCG) for first 20– 24 h followed by hormone free medium for next 20– 24 h can give better results for IVM of porcine oocyte than TCM-199 with ESS, PFF and hormone (PMSG+hCG) *in-vitro* maturation rates in oocytes of pigs reared under agro climatic hill ecosystem of Meghalaya.

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