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EFFECTS OF *IN VITRO* MATURATION MEDIA ON *IN VITRO* FERTILITY OF PORCINE OOCYTES AND EARLY DEVELOPMENT OF EMBRYOS

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SUMMARY

In pigs, embryo productivity is still lower than that in other livestocks. One of the reasons is incomplete maturation of porcine oocytes in *in vitro* conditions. Therefore *in vitro* maturation (IVM) plays a crucial role in *in vitro* production of porcine embryos. It provides prerequisite condition to in fertilization and subsequent development of porcine embryos. In a previous study, effects of NCSU-37-based medium and TCM-199-based media supplemented with porcine follicular fluid (pFF) or Fetal Bovine Serum (FBS) on *in vitro* maturation of Landrace oocytes collected in Vietnam have been compared, suggesting that NCSU-37 medium supplemented with 10% of porcine follicular fluid (pFF) had the highest rate of oocytes reach to metaphase II stage in comparison to those of the other two TCM-199-based media. In the present study, further experiments were carried out to evaluate the contribution of IVM media on fertilization capability and developmental competence. Porcine oocytes matured *in vitro* in 3 media: NCSU-37 supplemented with 10% pFF, TCM-199 supplemented with either 10% pFF or 10% FBS were subjected to *in vitro* fertilization and subsequent *in vitro* culture to monitor fertility and embryo development. The results showed that penetration and normal fertilization rates in both TCM-199 groups are both higher than that of NCSU-37 group. Moreover, the cleavage and blastocyst rates, and cell numbers of blastocysts which is a criterion for embryo quality were all higher in TCM-199 groups, especially in the group supplemented with pFF. It might be concluded that TCM-199 media supplemented with either pFF or FBS are suitable for effective *in vitro* maturation of Landrace porcine oocytes collected in Vietnam.

Keywords: *in vitro maturation, TCM-199, NCSU-37, pFF, FBS, in vitro fertilization, embryo development*

INTRODUCTION

In vitro maturation and *in vitro* fertilization has been successful since 1989s with piglets produced from IVM and IVF of porcine oocytes (Mattioli *et al.*, 1989). However, incomplete maturation of porcine oocytes in culture led to low embryo productivity in this species (Nagai *et al.*, 2006). Many researches were carried out to address this issue (Kątska-Książkiewicz, 2006), including testing of many medium systems and supplementation of necessary substances in order to improve the oocyte's situation after culture, in order to achieve better fertilization and embryo development (Nagai, 2001). Systems based on Tyrode's albumin lactate pyruvate medium (TALP) (Yoshida *et al.*, 1992), Tissue Culture Medium 199 - TCM 199 (Abeydeera and Day, 1997), North Carolina State University (NCSU) containing either taurine and hypotaurine (NCSU-23) (Li *et al.*, 2004; Margot *et al.*, 2001) or sorbitol - NCSU-

37 (Kikuchi *et al.*, 2002; Yoshioka *et al.*, 2003) were developed and all have achieved higher and higher fertilization rates, higher blastocyst rates and/or success of embryo transfer.

Supplementation of substances could contribute effectively to maturation of porcine oocytes. Supplementation of hormones helps significantly increase *in vitro* maturation rates as well as penetration of sperm into oocytes and rates of embryos reached to blastocyst stage (Funahashi and Day, 1989; Suzuki *et al.*, 2003). *In vitro* maturation rates varied by culture media, NCSU-23 could achieve a rate of 33.0-93.1% depends on oocyte quality (Quian *et al.*, 2001), NCSU-37 up to 90.5% (Spinaci *et al.*, 2008); TCM 199, Waymouth, mTLP-PVA, they were 61%, 64%, 70%, respectively, however, with pFF supplementation much better improvement could be revealed (92, 94 and 88%, respectively) (Yoshida *et al.*, 1992). Criteria of fertilization and embryo development, in general, are in proportional to maturation rates (Funahashi and Day, 1989; Quian *et al.*, 2001; Suzuki *et al.*, 2003; Kikuchi *et al.*, 2002; Yoshioka *et al.*, 2003). pFF supplementation contribute to expansion of cumulus cells and ooplasmic maturation, therefore fertilization is more well-prepared and more embryos could reach to higher stage of development (Kikuchi *et al.*, 2002; Marchal *et al.*, 2001; Yoshioka *et al.*, 2003).

In Vietnam, *in vitro* maturation and *in vitro* fertilization have been deployed since the 2000s (Nguyen, 2003; Duyen *et al.*, 2003). Effects of many factors, such as season, hormone supplementation, sperm concentration, feeder cell co-culture, etc. on IVM and IVF of porcine oocytes have been studied (Duyen *et al.*, 2003; Uoc *et al.*, 2008; Hiep *et al.*, 2014). Recently, Nguyen *et al.* (2015) while comparing maturation of Ban oocytes, could achieve a rate of 78.6% maturation in Landrace oocytes in NCSU-37 medium without hormone. However, the research did not survey on fertilization criteria. In a previous study, we evaluated IVM efficiency with 3 media: NCSU-37 supplemented with 10% of porcine follicular fluid (pFF) (Group 1 - control), TCM 199 supplemented with either 10% fetal bovine serum (FBS) (Group 2) or 10% pFF (Group 3). The results showed that Group 1 had the highest rate of oocytes reach to metaphase II stage in comparison to the other two groups, the rate of MII oocytes of TCM 199 supplemented with pFF is higher than that supplemented with FBS (Hiep *et al.*, 2018). In the present study, we continue to compare the three medium formulas in the aspect of support to *in vitro* fertilization and embryo development.

MATERIALS AND METHODS

Oocyte collection

Oocytes were collected as previously described (Kikuchi *et al.*, 2002, Hiep *et al.* 2018). Briefly, ovaries were collected from Landrace sows at a slaughter house in the suburb of Hanoi, rinsed and transported to the laboratory in physiological saline solution (0.9% NaCl) with antibiotics at 35˚C. The ovaries were rinsed several times in PBS and cumulus oocytes complexes (COCs) were aspirated from ovarian follicles using a scalpel blade. COCs were selected in TCM-199 medium with Hanks' salts supplemented with 5% FBS, 20 mM HEPES, 100 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate under a stereo microscope. COCs having 2 uniform layers of cumulus cells and a homogenous cytoplasm were selected for subsequent *in vitro* maturation.

In vitro maturation

Oocytes were randomLy divided into 3 groups of approximately 30-35 oocytes and cultured in 4-well dish containing 500 µL of maturation media supplemented with 0.6 mM cysteine, 50 µM β-mercaptoethanol (β-ME), 10µg/mL FSH and LH, and 1.0 mmol/L dibutyryl cyclic adenosine monophosphate (dbcAMP) at 390C, 5% CO2, 5% O2, 90% N2 in humidified air.

Group 1: COCs were cultured in NCSU-37 medium supplemented with 10% pFF.

Group 2: COCs were cultured in TCM-199 medium supplemented with 10% pFF.

Group 3: COCs were cultured in TCM-199 medium supplemented with 10% FBS.

After 20-22 hours, COCs in groups were then transferred into other disks containing the same medium but without hormones and dbcAMP for a further culture of 22-24 hours, before subjected to *in vitro* fertilization.

In vitro fertilization and embryo culture

In vitro fertilization and *in vitro* embryo culture (IVC) procedures were performed as described by Kikuchi *et al.* (2002). Briefly, COCs after IVM were washed and transferred into 90 µL drops of Pig-FM medium in cell culture disks covered with mineral oil. Epididymal sperms, collected and frozen as previously described (Hiep *et al.*, 2014), were washed, centrifuged and activated at 37ºC for 15 minutes in sperm washing medium (TCM-199 with Earle's salts, pH adjusted to 7.8) in a 30-mm petri dish, covered by paraffin oil. A suitable volume of sperms were diluted in Pig-FM medium after determining the concentration of sperm. 10 µL of the correspondent sperm dilution was introduced into the 90-µL IVF droplets containing the oocytes to a final concentration of 10^6 sperm/mL and co-incubated at 39ºC 38.5ºC under 5% CO_2 for 3 hours. At the end of IVF, spermatozoa and cumulus cells were removed from the surface of the *zona pellucida* by gentle pipetting with a fine glass pipette. Then, oocytes were either fixed for evaluation of fertilization, or cultured for 10 h in 500-µL drops of IVC-PyrLac and IVC-Glu for 2 and 4 days, respectively (Kikuchi *et al.* 2002) in 4-well dishes in an atmosphere of 5% CO2, in air at 38.5ºC. At day 2 and day 6 of *in vitro* culture (day of fertilization is defined as day 0), embryos were recorded for ones which could reach to cleavage and blastocyst stage (after checking by fixation, staining and cell number count), respectively.

Evaluation of fertilization

The fertilization status of oocytes was assessed 10 hours after IVF. Oocytes were mounted on glass slides and fixed with acetic alcohol (acetic acid 1 : ethanol 3) for at least 5 days, stained with 1% (w/v) orcein in acetic acid, rinsed in glycerol : acetic acid : water (1:1:3) and then examined under a phase-contrast microscope. The status of oocyte chromatin, the presence and numbers of female and male pronuclei and/or sperm head(s) and existence of the first and second polar bodies (1PB and 2PB, respectively) were investigated in the oocytes. Number of oocytes with penetration, ones which could form male pronuclear (MPN), and ones with normal fertilization defining as appearance of one female pronuclear, one male pronuclear, and two extruded polar body (Figure 1A), were recorded.

Evaluation of blastocyst quality by blastomere number

All embryos were fixed on glass slides at day 6 of culture in fixative containing ethanol : acid acetic with a ratio of 3 : 1 (v/v) for 3-4 days. Embryos were then stained with 1% (w/v) orcein in acetic acid, rinsed in glycerol : acetic acid : water (1:1:3). Blastomere numbers are counted by the cells nuclear which were stained red with orcein under phase contrast microscope. With each embryo, cell number was counted for 3 times and an average number of those times was used as the final result. An embryo was considered a blastocyst when it had more than 10 cells and a visible blastocoel in the cavity of it (Figure 1B).

Statistical analysis

Data were expressed as number and percentages were under the form of mean ± SEM values. Data were analyzed by one-way ANOVA on MS Excel Software.

RESULTS AND DISSCUSSION

Effects of *in vitro* maturation media to fertilization status of porcine oocytes are shown in Table 1. Rates of penetration, MPN formation in groups matured in TCM-199 with pFF and TCM-199 with FBS were 50.3% and 49.3%, and

94.8% and 97.1%, respectively, higher than those of NCSU-37 (25.4% and 69.8%) (P<0.05). There was no significant difference between the two TCM-199 formulas in penetration and MNP formation rates (P>0.05).

In TCM-199 with FBS group, normal fertilization rate was higher than that of group matured in TCM-199 with pFF (66.2% vs. 58.4%, P<0.05). In the meanwhile, normal fertilization rate of NCSU-37 was only 37.2%, significantly lower than those of the other groups (P<0.05).

Wang *et al.*, (1997) carried out a study in which the penetration rates of oocytes matured in NCSU-23, TCM-199 and mWM media were higher than in our study (71, 76, and 74%, respectively). However, MPN formation rates are equal to our study's (92, 83, and 86%, respectively). No difference was found between media and supplementation. Penetration and normal fertilization rates are much higher than those in our study. It might be because of differences in ovary sources between the two researches. In Vietnam, sows are usually slaughtered at a younger age than ones in developed countries, making ovaries collected at an earlier stage of maturation.

Besides fertilization status, we also checked the developmental competence of embryos produced from oocytes cultured in different medium formulas. The results are shown in Table 2. Cleavage rates were higher in TCM-199 groups than in NCSU-37 group (50.0%, and one supplemented with pFF was higher than one with FBS (74.0% vs. 70.0%). Blastocyst rates of Groups 1, 2 and 3 were 6.3%, 13.4%, and 19.6%), in which TCM-199 showed a significant advantage in formation of blastocyst in compare to NCSU-37, and FBS supplementation showed a significant advantage to pFF supplementation.

Table 1. Effects of *in vitro* maturation media to fertilization status of porcine oocytes

Group	Culture medium	Total number of oocytes	Number (%) of oocytes with penetration	Number (%) of oocytes with MPN	Number (%) of normal fertilization
1	NCSU-37 with 10% pFF	158	43 (25.4 ± 2.6)^{b}	30 (69.8 ± 8.0)^{b}	16 (37.2 ± 6.2)^{c}
2	TCM-199 with 10% pFF	94	68 (49.3 ± 2.7)^{a}	66 (97.1 ± 2.0)^{a}	45 (66.2 ± 6.4)^{a}
3	TCM-199 with 10% FBS	78	77 (50.3 ± 7.4)^{a}	73 (94.8 ± 4.7)^{a}	45 (58.4 ± 4.5)^{b}

5 replications were performed. Superscripts in the same column indicate significance (P<0.05).

Table 2. Effects of *in vitro* maturation media to development of *in vitro* produced porcine embryos

Group	Culture medium	Total number of oocytes	Number (%) of cleavage	Number (%) of blastocyst	Cell number of blastocysts
1	NCSU-37 with 10% pFF	158	79 (50.0 ± 3.5)^{b}	10 (6.3 ± 1.0)^{c}	(18.5 ± 2.3)^{b}
2	TCM-199 with 10% pFF	253	187 (74.0 ± 3.2)^{a}	34 (13.4 ± 1.9)^{b}	(28.5 ± 1.6)^{a}
3	TCM-199 with 10% FBS	266	186 (70.0 ± 6.8)^{a}	52 (19.6 ± 1.2)^{a}	(31.7 ± 1.5)^{a}

5 replications were performed. Superscripts in the same column indicate significance (P<0.05). Cell number is performed as mean ± SEM.

Similarly in Wang *et al.* (1997), effects of *in vitro* maturation media on development of porcine embryos were also studied. Oocytes matured in NCSU-23, TCM-199 and mWM were *in vitro* fertilized and cultured. At 48 hour post insemination (h.p.i), cleavage rates reached 61-70% (NCSU-23: 70%, TCM-199 and mWM: 61%), which were equal to that of the present study. At day 6 of embryo culture, blastocyst rate of group using NCSU-23 medium were significantly higher than those in TCM-199 and mWM (27%, 15%, and 4%, respectively). Average cell number in each groups were 36.8, 30.7, and 29.4, respectively. NCSU-23 showed advantages in both blastocyst rate and cell number in compare to TCM-199 and mWM. NCSU-23, a little different version of NCSU-37, was a popular medium for *in vitro* maturation applying in many previous researches (Abeydeera and Day, 1997; Karja, 2008; Suzuki *et al.*, 2003). The results in our study were in contrast of that previous study, in which TCM-199 medium and FBS supplementation showed a better contribution to development of embryos to cleavage and blastocyst stages. Average cell numbers in the present study are not significant between groups, and all is similar and even more stable to those in Wang *et al.* (1997).

In the previous study, we had shown that NCSU-37 contributed better to *in vitro* maturation of Landrace porcine oocytes than TCM-199 supplemented with either pFF or FBS (Hiep *et al.*, 2018). However, the results in this study showed that better maturation, in the form of completion of first meiosis, did not in accordance with fertility, in the form of penetration, MPN formation and normal fertilization, and development competence, in the form of cleavage, blastocyst formation, and blastocyst cell number. TCM-199 group had higher criteria of fertilization and development in compare to NCSU-37, and FBS supplementation was equal to better than pFF supplementation. FBS and pFF both have their own advantages and disadvantages. With pFF, it is cheap and easy to harvest, however, it is complicated to be treated and to be kept from contamination. With FBS, it is commonly used in cytology and embryology with commercialized various products, however, it is rather more expensive than pFF.

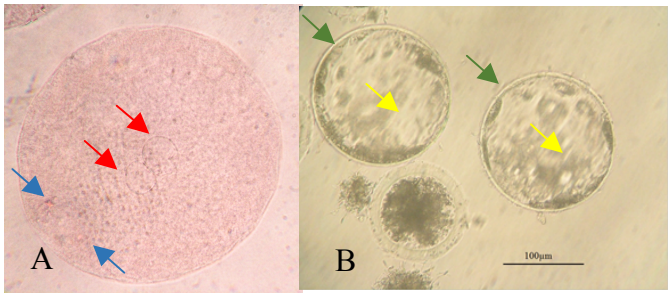

Figure 1. (A) A normally fertilized oocyte at 400X magnification with a male pronuclear, a female pronuclear (red arrows), and two polar body extruded (blue arrows); and (B) porcine embryos at blastocyst stages (green arrows) with blastocoels (yellow arrows).

CONCLUSION

TCM-199 medium for maturation contribute to penetration, fertilization and subsequent embryo production better than NCSU-37 medium. Embryos produced by oocytes cultured in maturation medium supplementation with FBS could reach to blastocyst stage with a higher rate than that with pFF.

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ẢNH HƯỞNG CỦA MÔI TRƯỜNG NUÔI THÀNH THỤC LÊN KHẢ NĂNG THỤ TINH ỐNG NGHIỆM CỦA TRỨNG LỢN VÀ SỰ PHÁT TRIỂN PHÔI SỚM

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TÓM TẮT

Hiệu suất tạo phôi ở lợn vẫn còn thấp khi so sánh với ở các loài khác. Một trong những nguyên nhân chủ yếu là trong điều kiện *in vitro* trứng lợn không phát triển thành thục hoàn thiện như ở các loài khác. Vì vậy, nuôi thành thục *in vitro* đóng một vai trò đặc biệt quan trọng trong việc sản xuất phôi lợn thụ tinh ống nghiệm. Nuôi thành thục *in vitro* tạo điều kiện thích hợp cho sự thụ tinh cũng như sự phát triển của phôi. Trong nghiên cứu trước đây, ảnh hưởng của môi trường NCSU-37 và môi trường TCM-199 có bổ sung dịch nang trứng lợn (pFF) hoặc huyết thanh thai bò (FBS) lên sự thành thục *in vitro* của trứng lợn Landrace thu thập ở Việt Nam đã được so sánh, cho thấy môi trường NCSU-37 bổ sung 10% of pFF có tỷ lệ trứng đạt đến giai đoạn gian kỳ II cao nhất so với hai loại môi trường TCM-199. Trong nghiên cứu này, những thí nghiệm tiếp theo được tiến hành để xác định đóng góp của môi trường nuôi thành thục lên khả năng thụ tinh và phát triển phôi. Trứng lợn được nuôi trong 3 loại môi trường: NCSU-37 bổ sung 10% pFF, TCM-199 bổ sung 10% pFF hoặc 10% FBS, sau đó được thụ tinh và đưa vào nuôi để theo dõi trạng thái thụ tinh và phát triển phôi. Kết quả cho thấy tỷ lệ tinh trùng xâm nhập và tỷ lệ thụ tinh bình thường ở các nhóm trứng nuôi thành thục bằng môi trường TCM-199 đều cao hơn của nhóm nuôi bằng môi trường NCSU-37. Tỷ lệ phôi phân chia và phôi nang cũng như số tế bào của phôi nang - một tiêu chí để đánh giá chất lượng phôi - đều cao hơn ở các nhóm trứng nuôi thành thục bằng môi trường TCM-199, đặc biệt là nhóm được bổ sung pFF. Do đó, môi trường TCM-199 bổ sung pFF hoặc FBS phù hợp để nuôi thành thục *in vitro* một cách hiệu quả đối với trứng lợn Landrace thu nhận ở Việt Nam.

Từ khóa: *nuôi thành thục in vitro, TCM-199, NCSU-37, pFF, FBS, thụ tinh ống nghiệm, phát triển phôi*