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

Nguyen Viet Linh¹,²,*, Nguyen Thi Hiep¹

¹Institute of Biotechnology, Vietnam Academy of Science and Technology
²Graduate University of Science and Technology, Vietnam Academy of Science and Technology

*To whom correspondence should be addressed. E-mail: nvlinh@ibt.ac.vn

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 (Kataska-Ksiazkiewicz, 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-
Nguyen Viet Linh & Nguyen Thi Hiep

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 dishes 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 10⁶ sperm/mL and co-cultivated at 39ºC 38.5ºC under 5% CO₂ 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 DISCUSSION**

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)                        | 30 (69.8 ± 8.0)               | 16 (37.2 ± 6.2)                    |
| 2     | TCM-199 with 10% pFF    | 94                      | 68 (49.3 ± 2.7)                        | 66 (97.1 ± 2.0)               | 45 (66.2 ± 6.4)                    |
| 3     | TCM-199 with 10% FBS    | 78                      | 77 (50.3 ± 7.4)                        | 73 (94.8 ± 4.7)               | 45 (58.4 ± 4.5)                    |

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)         | 10 (6.3 ± 1.0)           | (18.5 ± 2.3)              |
| 2     | TCM-199 with 10% pFF    | 253                     | 187 (74.0 ± 3.2)        | 34 (13.4 ± 1.9)          | (28.5 ± 1.6)              |
| 3     | TCM-199 with 10% FBS    | 266                     | 186 (70.0 ± 6.8)        | 52 (19.6 ± 1.2)          | (31.7 ± 1.5)              |

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 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. 

Acknowledgement: The authors would like to thank the colleagues of the Laboratory of Embryo Technology, IBT, for assisting in experiments. The study is funded by Vietnam
REFERENCES

Abeydeera LR, Day BN (1997) Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod 57: 729–734.

Duyen HTL, Ngoc PK, Duong HHT, Dat NQ (2003) Application of in vitro fertilization technique on pigs. Proc Vietnam Nat Conf Biotech: 639-642.

Funahashi H, Day BN (1993) Effects of the duration of exposure to hormone supplements on cytoplasmic maturation of pig oocytes in vitro. J Reprod Fertil 98: 179-185.

Hiep NT, Thinh NH, Hanh NV, Viet Linh N (2018) Comparison of in vitro maturation of porcine oocytes using different maturation media. J Sci Tech Husbandry 232: 78-82.

Hiep NT, Uoc NT, Mai HN, Viet Linh N (2014) The effect of frozen sperm factor on developmental competence of porcine oocytes matured and fertilized in vitro. Thai Nguyen Univ J Sci Tech 123: 101-106.

Karja NWK (2008) Nuclear Maturation of Porcine oocytes in vitro: Effect of the Cumulus-Oocyte Complexes Quality. Indonesian J Biotech 13: 1078-1084.

Kążys-Książkiewicz L (2006) Pig embryo production by in vitro maturation and fertilization of ovarian oocytes. A review. J Anim Feed Sci 15: 525-542.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, Akita T, Nagai T (2002) Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biol Reprod 66: 1033-1041.

Li Q, Niwa K, Hunter MG (2004) Effects of 17 beta-estradiol on in vitro maturation of pig oocytes in protein-free medium. J Reprod Dev 50: 305-313.

Margot A, Nunes D, Charles G (2001) Influence of hormones and follicular fluid on maturation of pig oocytes. Ciência Rural, Santa Maria, 31 (1): 99-104.

Mattioli M, Bacci mL, Galeati G, Seren E (1989) Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology 31: 1201-1207.

Nagai T (2001) The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 55: 1291-1297.

Nagai T, Funahashi H, Yoshioka K, Kikuchi K (2006) Update of in vitro production of porcine embryos. Front Biosci 11: 2565-2573.

Ngyuen BX (2003) Development of embryo and embryonic cell technology in Vietnam. Proc Inst Biotech: 411-417.

Ngyuen BX, Kikuchi K, Uoc NT, Dang-Nguyen TQ, Linh NV, Men NT, Nguyen TT, Nagai T (2015) Production of Ban miniature pig embryos by in vitro fertilization: a comparative study with Landrace. Anim Sci J 86: 487-493.

Qian Y, Shi WQ, Ding JT, Fan BQ, Fuku Y (2001) Effect of follicle size on Cumulus - Expansion, In vitro fertilization and development of porcine follicular oocytes. J Reprod Dev 47: 145-152.

Spinaci M, Volpe S, De Ambrogi M, Tamanini C, Galeati G (2008) Effects of epigallocatechin-3-gallate (EGCG) on in vitro maturation and fertilization of porcine oocytes. Theriogenology 69: 877-885.

Suzuki H, Saito Y, Kagawa N and Yang X (2003) In vitro Fertilization and Poly spermyn in the Pig: Factors Affecting Fertilization Rates and Cytoskeletal Reorganization of the Oocyte. Microres Res Tech 61: 327-334.

Uoc NT, Ty LV, Duc NH, Chi BL, Thanh NT, Linh NV, Hanh NV, Huu QX, Anh NT, Son HN, Long DD, Nguyen BX (2003) “Production of dairy calves by in vitro fertilization and transfer of sex predetermined embryos. Proc Vietnam Nat Conf Biotech: 717-719.

Wang WH, L Abeydeera R, Cantley TC, Day BN (1997) Effects of Oocyte maturation media on development of pig embryos produced by in vitro fertilization. J Repro Fertility 111: 101-108.

Yoshida M, Ishigaki K, Pursel VG (1992) Effect of maturation media on male pronucleus formation in pig oocytes matured in vitro. Mol Reprod Dev 31: 68–71.
NHƯ HƯỠNG CỦA MÔI TRƯỜNG NƯỚI THÀNH THỰC LÊN KHÁ NĂNG THỰC TINH OfSize 15 trong TRONG LỒN VÀ SỰ PHÁT TRIỂN PHtíh SỐM

Nguyễn Việt Linh1,2, Nguyễn Thị Hiệp1

1Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
2Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÔM TÁT

Hiệu suất tạo phôi ở lớn vẫn còn thấp khi so sánh với ở các loại khác. Một trong những nguyên nhân chủ yếu là trong điều kiện in vitro trứng lớn không phát triển thành thực hoàn thiện như ở các loại khác. Vì vậy, nuôi thành thực in vitro dòng một vài trở được biết quan trọng trong việc sản xuất phôi lớn thực tình ông nghiêm. Nuôi thành thực in vitro tạo điều kiện thích hợp cho sự thịnh cư như sự phát triển của phôi. Trong nghiên cứu trước đây, anh hướng của môi trường NCSU-37 và môi trường TCM-199 có bộ sung dịch nang trứng lớn (pFF) hoặc huyết thanh bò (FBS) lên sự thành thực in vitro của trứng lớn Landrace thu thập ở Việt Nam đã được so sánh, cho thấy môi trường NCSU-37 bộ sung 10% của pFF có tỷ lệ trứng đạt đến giai đoạn gan 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 động gôp của môi trường nuôi thành thực lên khả năng thịnh và phát triển phôi. Trứng lớn được nuôi trong 3 loại môi trường: NCSU-37 bộ sung 10% pFF, TCM-199 bộ sung 10% pFF hoặc 10% FBS, sau đó được thử tình và dựa vào nuôi để theo dõi trạng thái thịnh và phát triển phôi. Kết quả cho thấy tỷ lệ trứng xâm nhập và tỷ lệ thịnh con thường ở các nhóm trứng nuôi thành thực bằng môi trường TCM-199 đều cao hơn của nhóm nuôi bằng môi trường NCSU-37. Tỷ lệ phôi phân chia và phát triển các cũng như số tế bào của phôi nảng - một tiêu chí để đánh giá chất lượng phôi - đều cao hơn ở các nhóm trứng nuôi thành thực bằng môi trường TCM-199, đặc biệt là nhóm được bộ sung pFF. Do đó, môi trường TCM-199 bộ sung pFF hoặc FBS phù hợp để nuôi thành thực in vitro một cách hiệu quả đối với trứng lớn Landrace thuần ở Việt Nam.

Từ khóa: nuôi thành thực in vitro, TCM-199, NCSU-37, pFF, FBS, thụ tinh ông nghiêm, phát triển phôi