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Effects of Caffeine on *In Vitro* Fertilization of Pig Follicular Oocytes

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Abstract

In the present study, experiments were conducted to assess the effects of caffeine on *in vitro* fertilization of pig follicular oocytes. Cumulus-oocyte complexes (COCs) were collected from porcine ovaries from slaughterhouses, cultured in *in vitro* maturation medium 1 (IVM1) at 38.5^{o}C for 20-22 hours and then in *in vitro* maturation medium 2 (IVM2) for the next 24 hours. Only the oocytes with expanded cumulus cells were selected for *in vitro* fertilization. Boar frozen semen was used for the porcine IVF procedure. The spermatozoa were pre-incubated in modified TCM 199 medium and subsequently incubated for 3 hours in porcine fertilization medium (pig FM) supplemented with either 2 mM or 5 mM of caffeine. They were cultured in IVC1 medium supplemented with pyruvate and lactate from day 0 to day 2, and then in IVC2 medium supplemented with glucose from day 2 to day 6. The results indicate that pig FM containing 5 mM caffeine gave a higher penetration rate than 2 mM caffeine (33.4% vs. 11.4%, respectively). The blastocyst rates of the two groups were not significantly different (8.4% and 8.0%). In conclusion, a higher caffeine concentration in the fertilization medium may ensure the production of *in vitro* porcine embryos with acceptable productivity utilizable for further studies on this subject.

Keywords

Caffeine, pig oocytes, *in vitro* fertilization, IVM, IVC.

Introduction

Along with the strong and rapid development of the economy, animal breeding, especially pig breeding, is constantly developing and there have been many significant changes in recent years. Pigs have an important role in providing food and are a good model to perform studies because they have many biological similarities to

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humans. Porcine embryos production by *in vitro* fertilization (IVF) has been studied since the early 20th century (Cheng *et al.*, 1986). *In vitro* production of embryos is important because of its high efficiency in creating large quantity of embryos with good quality and viability. Compared to the *in vivo* method, the *in vitro* production method could minimize the use of sows in experiments because with ovaries obtained from slaughterhouses, a large quantity of carefully selected oocytes can be collected for experiments; therefore, the cost of embryos is also considerably lower. Many studies have been performed on *in vitro* production of pig embryos, however its productivity is still low. In published experiments, caffeine, a methyl xanthine, was shown to be essential for the induction of the acrosome reaction, which results in the penetration of oocytes by sperm, and at the same time, stimulates IVF, greatly reduces the time of fertilization, and promotes faster penetration of sperm into the oocytes (Nagai *et al.*, 1993). Different caffeine concentrations in the medium have certain influences to the embryo rate and the penetrated rate. In Vietnam, pigs are often slaughtered at 4-5 months old which is prior to mature age, and is earlier than in the developed countries. Because of this, pig oocytes usually are of a lower quality. Therefore, we carried out this study to determine the effects of caffeine on *in vitro* fertilization of pig follicular oocytes in order to improve the *in vitro* production (IVP) system in Vietnam.

Materials and Methods

Materials

Pig ovaries of Landrace were collected from a slaughterhouse in Thanh Oai, Hanoi for oocyte collection. Landrace frozen sperm were collected and preserved in liquid nitrogen at the Laboratory of Embryo Technology, Institute of Biotechnology, Vietnam Academy of Science Technology.

Ovaries were cut off immediately after slaughtering and quickly transferred to the laboratory at 37ºC. Thereafter, the ovaries were washed twice in physiological saline solution containing antibiotics. Follicles 3-6 mm diameter were selected for oocyte collection (Nagai *et al.*, 1988). Follicular fluid from the follicles with appropriate diameters were collected with a 10 mL syringe, 20G needle. Oocytes in the fluid were selected and obtained under a stereo microscope. Only oocytes of quality A and B (uniformly dark cytoplasm, with an even 3-5 layers of cumulus cells) were collected for the experiment.

In vitro maturation

The cumulus-oocyte complexes (COCs) were gently rinsed and then cultured in IVM1 medium (TCM 199 supplemented with 10% pFF, 0.6 mM L-cysteine, 0.2 mM Na-pyruvate, 50 mM β-mercaptoethanol, dbcAMP, hormones) at 38.5ºC, 5% CO_2, and saturated humidity. After 20-22 h, oocytes were transferred to a dish with IVM2 medium (TCM 199, pFF, L-cysteine, Na-pyruvate, β-mercaptoethanol) for further incubation of another 24 h. After *in vitro* maturation, oocytes were used for *in vitro* fertilization.

In vitro fertilization

After maturation, oocytes were removed from the cumulus by gentle pipetting in 100 µL *Hyaluronidase* and incubated with frozen-thawed sperm at a concentration of 10^5 sperm mL^{-1} for 3 h at 38.5ºC, 5% CO_2, and saturated humidity, in a fertilization medium (Kikuchi *et al.*, 2002) with either 2 mM or 5 mM caffeine.

Oocytes were then washed twice in IVC-PyrLac medium to remove residual cumulus cells and attached sperm, and incubated in IVC-PyrLac medium for 2 days and in IVC-Glu medium for the next 4 days.

Evaluation of fertilization and embryo development

Blastocysts on day 6 after fertilization were fixed and dipped into a solution of 3 parts ethanol to 1 part acid acetic for 3-7 days. Then, sample fixation was dyed by 1% aceto-orcein for 5-7 min and observed under a stereo microscope.

Statistical analysis

Data were analyzed by Minitab 18 software and single factor ANOVA by Microsoft Excel version 2013.

Results and Discussion

Effect of caffeine concentration in the fertilization medium on *in vitro* fertilization

From Table 1, it can be seen that the penetrated oocyte rates and polyspermic rates of porcine fertilization medium (pig FM) supplemented with 5 mM caffeine were higher than in the 2 mM caffeine treatment, respectively (33.4% and 11.4%, 18.9% and 5.6%, respectively). The monospermic rates of both groups were significantly higher than the polyspermic rates. The penetrated oocyte rates, monospermic rates, and polyspermic rates were not significantly different ($P>0.05$) between the 2 mM caffeine group and the 5 mM caffeine group.

Nagai *et al.* (1993) reported that in *in vitro* fertilization in pig FM with 2 mM caffeine, the penetrated oocyte rate was 62% (29 penetrated oocytes of 47 matured oocytes), and with a caffeine concentration of 5 mM, the penetrated oocyte rate was 83% (40 penetrated oocytes in 48 matured oocytes), much higher than the rates we achieved in the present study. One of the factors that influences the quality of pig oocytes matured *in vitro* is the stage of maturity of the sows. In Vietnam, pigs are slaughtered at 4 - 5 months old, not yet mature, which is earlier than in the developed countries. Because of this, pig oocytes obtained for the study might have an inferior quality with lower penetration rates. However, our results are in accordance with Nagai's *et al.* (1993) in the aspect of promoting penetration with a higher (5 mM) caffeine concentration.

Effect of caffeine concentration in the fertilization medium on *in vitro* pig embryo development

As shown in the Table 2, there is no obvious difference between the blastocyst rates of 2 mM caffeine and 5 mM caffeine (8.4% and 8.0%). The cleavage rates and the morulae rates were lower in the group with 5 mM caffeine in pig FM than that of the group with 2 mM (37.0% and 55.8%, 58.1% and 63.6%, respectively). This result corresponds to the influence effect of caffeine concentration on the fertilization medium for *in vitro* fertilization in Table 1, as fertilization is crutial for the formation and development of embryos.

Thoa *et al.* (2009) reported that pig embryo production with 2 mM caffeine and achieved a morula rate of 63.5% and blastocyst rate of 13.6% with grade A oocytes. Similarly, Suzuki *et al.* (2006) obtained a blastocyst rate of 19%. In our current study, the morula and blastocyst rates were lower in both the 2 and 5 mM caffeine groups. This might be due to the quality of the samples as aforementioned and the selection of oocytes for fertilization (we used both grade A and B oocytes). Futher evaluation, such as blastocyst cell number, may reflect the quality of embryos, i.e. productivity of the system.

Table 1. Effect of caffeine concentration in the fertilization medium on *in vitro* fertilization

Concentration of caffeine	No. of oocytes examined	No. of fertilized oocytes	Penetration rates (%)	No. of monospermic oocytes	Monospermic rates (%)	No. of polyspermic oocytes	Polyspermic rates (%)
2 mM	160	19	11.4 ± 4.1[a]	18	94.4 ± 5.6	1	5.6 ± 5.6
5 mM	145	51	33.4 ± 8.0[b]	38	81.1 ± 10.1	13	18.9 ± 10.1

Note: 03 replications were carried out. Data are presented as mean ± S.E.M. In a column, data with different superscripts are significantly different (P>0.05).

Table 2. Effect of caffeine concentration in fertilization medium on embryo *in vitro* development

Concentration of caffeine	No. of oocytes examined	No. of cleavage embryo	Cleavage rates (%)	No. of morulae	Morulae rates (%)	No. of blastocysts	Blastocyst rates (%)
2 mM	207	131	63.6 ± 3.0	71	55.8 ± 9.8	11	8.4 ± 1.8
5 mM	210	121	58.1 ± 4.2	44	37.0 ± 3.0	10	8.0 ± 3.7

Note: 04 replications were carried out. Data was presented as mean ± S.E.M.

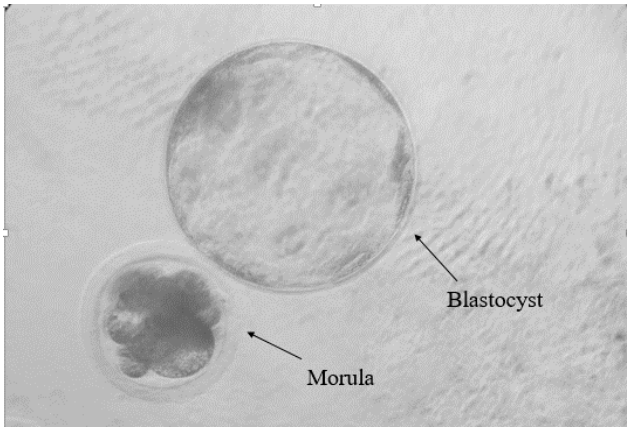
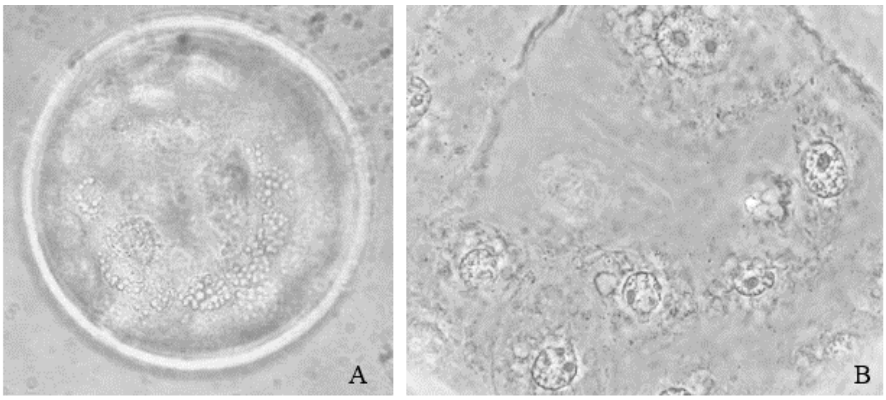


Figure 1. A blastocyst on day 6 after fertilization (magnification 200X)



Note: A – Blastocyst (magnification 200X) and B – Cells in the blastocyst (magnification 400X).

Figure 2. Cells in a blastocyst (Blastomere)

Conclusions

Fertilization medium supplemented with 5 mM caffeine had higher penetration rates than medium supplemented with 2 mM caffeine, however, blastocyst rates between the two groups were not significantly different.

Supplementation with a higher concentration of caffeine had a significant effect on improving penetration during *in vitro* fertilization of pig oocytes, however, there was no improvement in the ability to form blastocysts. The results of this study could contribute to the improvement of the IVP system from slaughter houses in Vietnam.

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