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# Effect of Insulin Supplement on Development of Porcine Parthenogenetic Embryos

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## ABSTRACT

This study is performed to evaluate the effect of insulin in the porcine parthenogenetic embryo development. In porcine embryo culture, insulin is helpful factor in the process of embryo development. To identify this, insulin is used in pig embryos development. Therefore, this study was performed to investigate the effect of insulin on early embryonic development in pigs. For that, insulin positive or negative (0, 10 ug/mL) was supplemented in the porcine IVM media and then compared two groups divided by the cytoplasm of the black groups and white ring groups based on the distribution of lipid material of the cell cytoplasm in microscope. In maturation rates of porcine oocytes, significant higher black group rates were shown in the insulin positive groups compared with other groups (56.0±2.1 vs 46.2±0.3). In the embryo culture, black groups were showed the significant higher cleavage rates (82.1±0.8, 78.3±0.1 vs 63.2±0.3, 63.4±0.0), and blastocyst formation rates (15.5±3.6, 16.6±0.4 vs 11.7±1.3, 7.4±0.2) regardless of whether the addition of insulin. Also, black groups were showed higher cell number of blastocyst (33.2±2.5, 35.5±2.6 vs 31.2±2.1, 31.3±2.2). In conclusion, supplement of insulin producing black group in vitro maturation, it was effective *in vitro* maturation and embryonic development of pig embryos.

(Key words: Porcine, Embryo, *in vitro* production, Insulin)

## INTRODUCTION

Recently, *in vitro* production (IVP) of porcine embryo is in broad researched for production technique and embryo genesis research. These days porcine embryo researches are expand to biomedical research and xenotransplantation part (Yoshioka 2011). Especially, the pig is considered an appropriate biomedical model for human study, in special organ development and disease progression, because it is similar physiology and size (Lunney 2007).

Dolly the sheep was first cloned animal mammal successfully produced by enucleated oocytes with mammalian cell (Wilmut et al 1997). In miniature pig, many studies have been performed which has many similarities with human (Sugimura et al 2010, Wakai et al 2008, Yamanaka et al 2009, Yamanaka et al 2007) and in SCNT reported that endangered species preservation, cell therapies, livestock propagation, short life span, abnormal growth with low efficiency. For production of offspring in pig, buffalo and mouse culture environment is very important for post-activation development, blastocysts formation and gene expression (Thirthagiri et al 2009, Wadhwa et al 2009, Yamanaka et al 2009). SCNT embryos production (Wilmut et al 1997) were more challenging than *in vitro* fertilized (IVF) and parthenogenesis embryos in culture media for formation of blastocysts quality (Yamanaka et al 2009). For development of culture media many researchers have been tried supplement of amino acids (Gupta et al 2008), glucose (Han et al 2008), pyruvate (Lee et al 2003) and growth factors (Pandey et al 2009). Blastulation, hatching rates, post-implantation development were depend on drop size and density of embryos culture (Salahuddin et al 1995, Vutyavanich et al 2011). As a result, some factors such as insulin growth factors, transforming growth factors (TGFs), epidermal growth factors (EGFs) (Dadi et al 2007), fibroblast growth factor, platelate-activating growth factors (Lee et al 2004) and exogenous growth factors for embryonic development (Biswas et al 2011). Some researchers have been reported that, growth factors have beneficial effects on embryonic development in culture media (Dadi et al 2007,

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ONeill 1997).

The capability of porcine oocytes to *in vitro* mature, fertilize, and develop has been proved by several researchers, and successful methods for *in vitro* maturation and fertilization of porcine oocytes and culture of porcine embryos have been developed. But, even if pig oocytes develop to blastocyst stages by fertilization *in vitro* developmental potential is higher than *in vivo* (Day 1993).

For development and follicular survival insulin and FSH were more crucial in in vitro culture media (Hartshorne et al 1997). During growth and maturation within ovarian follicles some growth factors were important in mammalian oocytes such as epidermal growth factor (EGF), insulin like growth factor 1 (IGF-1), and insulin (van den Hurk & Zhao 2005).

Insulin in an anabolic and peptide hormone which is produced in islets of Langerhans in the pancreas by β-cells. By intake food levels of insulin secretion is increased by free fatty acid (FAF) and amino acid concentration in blood (Freret et al 2006). Insulin is more important because of enhance nutrients in cells and synthesis of proteins and triglycerides (Sjaastad et al 2003).

Follicular/luteal phase varies approximately 0.1-10 ng/mL depends on insulin levels (Adamiak et al 2005, Spicer & Echternkamp 1995). Similarly, plasma levels were same and low levels concentration found in follicular fluid when compared with plasma (Landau et al 2000, Spicer & Echternkamp 1995). Insulin would be helpful for systemic nutritional status and ovary by levels follicular fluid to plasma levels (Spicer & Echternkamp 1995).

*In vitro* oocytes maturation, in pre-ovulatory follicles insulin was high when compared with subordinate follicles because of insulin effect (Landau et al 2000). Granulosa cells of ovarian proliferation and P4 production enhanced by insulin and steroidogenesis of luteal cells were increased in different species (Spicer & Echternkamp 1995). Insulin may affect the steroidogenesis, glucose metabolism and cells growth or differentiation but not the target tissue so that normal ovarian physiology and ovarian functions disorder were closely related with insulin stimulation. Oocytes maturation were controlled by insulin and gonadotrophins after providing energy intake of glucose and granulosa cells glycolysis. For improvement of parthenogenetic oocytes some chemical reagents were used in reconstructed oocytes such as cytochalasins B, demecolcine, latrunculin A (Himaki et al 2012).

In this study, it was investigated for the influence of insulin on comparison with and without additional supplement in maturation media on porcine embryonic development. Within the period of research determined the maturation, embryonic development and quality of blastocysts of porcine embryos contrast to different media.

## MATERIALS and METHODS

### 1. *In vitro* maturation of porcine oocytes

All reagents and chemicals were purchase from Sigma-Aldrich Chemical company (ST. Louis, MO, USA) unless otherwise. Pig ovaries were collected from the local slaughter house and transported to the laboratory within 4 h washed with pre warmed normal 0.9% saline water. By using 18 gauges' needle syringe for follicle aspiration which were 3-8 mm in diameter. The aspirate was then collected in 15 mL conical tube and kept for 5min to settle as sediment. Supernatant fluid was then discarded by pipetting and collect oocytes in 60 mm dishes and placed in under microscope. COCs were selected with three layers of cumulus cells with distinct nucleus. Washed selected oocytes three times in the HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA; (Bavister et al., 1983) and cultured in maturation medium (TCM-199-Gibco) supplemented with 10 IU/mL of eCG/hCG, 0.2mM sodium pyruvate, 0.6mM cysteine, 0.91 mM pyruvate, 15 μg/mL kanamycine, 15 μg/mL epidermal growth factor, 10% (v/v) porcine follicular fluid (Song et al 2009) and placed the oocytes in 4 well multi-dishes. And oocytes were untreated or treated with 10 μg/mL insulin for 22 h humidified at 39°C with 5% $CO_2$. After 22 hours of maturation medium, oocytes were transfer hormone free medium and culture at another 18-21 h humidified at 39°C with 5% $CO_2$. Total incubation of 40-41 h of incubation removed the oocytes with 0.1% (w/v) hyaluronidase. The oocytes which have first polar body were selected for parthenogenic activation.

### 2. Production of parthenogenetic embryos

Reconstructed oocytes were activated with 2 pulses of 120 V/cm of direct electrical activation current for 60 μsec in 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$ (BTX, ECM, 2001). After activation, activated embryos were incubated 4 h in M-199 solution containing 0.4 μg/ml demecolcine and 2 mM 6-Dimethylaminopurine.

Parthenogenetic embryos were transfer to the PZM5 media 25 μl drops embryos were cultured in PZM-5 and covered by mineral oil. Parthenogenetic embryos conduct at 39°C, 5% $CO_2$, 5% $O_2$ at saturating humidity. Development of cleavage rates and rates of blastocysts formation were checked at day 2 and day 6; respectively.

### 3. Experimental plans

In this study compared of untreated or treated with 10 μg/mL insulin during IVM in porcine embryos. Experiments were performed 8 replicates and each experiments were used 100 oocytes. Maturation rates were evaluated after denuding oocytes which had a visible first polar body were taken for parthenohenetic activation and divided into two groups based on the lipid material in the cytoplasm; black group (Fig. 1) and white ring group (Fig. 2). Then activated embryos were post-activated for 4 h in M-199 solution containing 0.4 μg/ml demecolcine and 2 mM 6-Dimethylaminopurine. Cultured in PZM-5 media for further development. Controls were cultured in PZM-5 media after post-activation without giving any treatment. At day 2 and day 6 oocytes were examined on embryonic development of cleavages rates, blastocysts rates, respectively. Total blastocysts cells were counted under epifluorescent microscope by using Hoechst 33342 media.

### 4. Statistical analysis

Experimental data were analyzed by Statistical Analysis System (SAS, version 21.0; Windows, USA) with mean± SEM whereas $P$ value < 0.05.

## RESULTS

### 1. Effects of insulin on embryonic development *in vitro* maturation of porcine oocytes

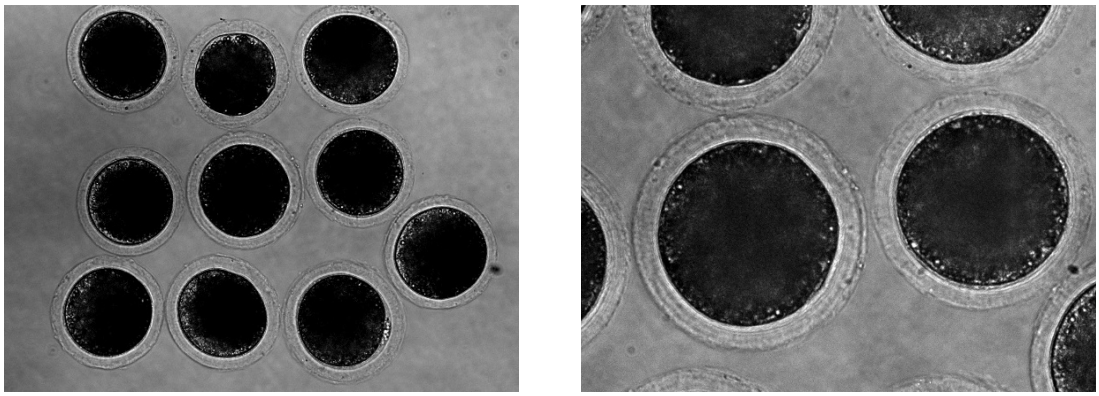

Fig. 1. The oocyte is filled with lipid material, cell cytoplasm looks a black in microscope. Microscope of 100 magnification (left), and 200 magnification (right) in black groups.

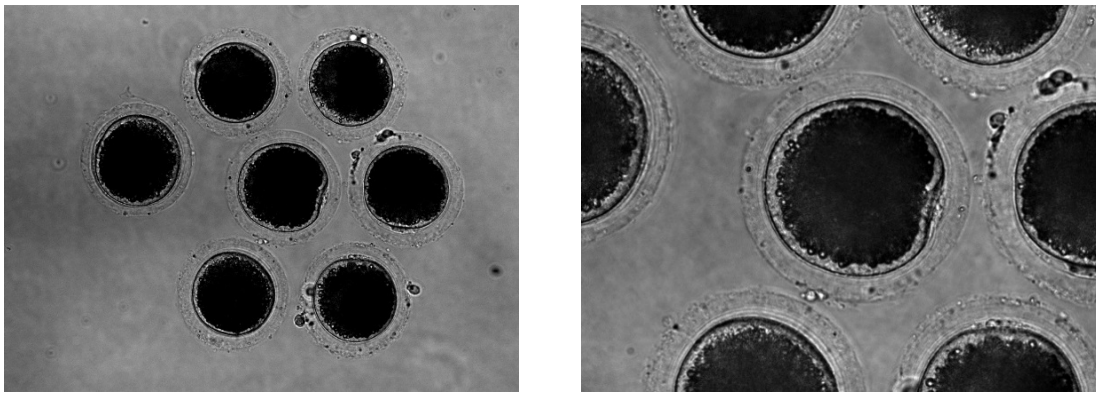

Fig. 2. The oocyte is less filled with lipid material compared to black groups (Fig. 1). The rim of cell cytoplasm looks white, so it looks like white rings in microscope. Microscope of 100 magnification (left), and 200 magnification (right) in white ring groups.

Table 1 shows the maturation of oocytes in IVM was evaluate by determining first polar body extrusion and calculate the maturation rates of the oocytes in separately in replicates. The black groups, oocytes filled with lipid material that cell cytoplasm looks a black in microscope, appear more than white ring groups, oocytes less filled with lipid material compared to black groups that the rim of cell cytoplasm appears white so it looks like white rings in microscope.

Effect of insulin in black group maturation rates (56.0%) were significantly higher white rings group (34.0%) but there were no significantly in both non treatment groups (46.2%, 44.5%, respectively).

### 2. Developments of porcine parthenogenetic embryos

Examined the development of parthenogenetic embryos with insulin positive, negative, and cytoplasm with black groups, white ring groups. Table 2 shows that the cleavage rates and rates of formation was significantly higher in black groups of insulin. Cleavage rates and blastocyst rates were significantly higher (82.1% vs 63.2% and 15.5% vs 11.7%; respectively) with insulin treatment. Similarly, without insulin in black group oocytes were significantly increased than white rings group (78.3% vs 63.4% and 16.6% vs 7.4%; respectively) on embryo cleavage rates and blastocyst formation rates.

### 3. Blastocysts cell number of porcine parthenogenetic pig oocytes

Examined the total cells in the parthenogenetic blastocysts with insulin positive, negative, and cytoplasm with black

Table 1. Effects of insulin treatment during embryonic development *in vitro* maturation of porcine oocytes

| | No. of embryos (%) | |
|---|---|---|
| Treatment of insulin | Cultured | Matured (%) |
| Insulin (+) Black[a] | | 224(56.0±2.1) |
| Insulin (+) White ring[b] | 400 | 136(34.0±0.7) |
| Insulin (-) Black | | 185(46.2±0.3) |
| Insulin (-) White ring | 400 | 178(44.5±0.1) |

8 replicates were performed.

[a]Oocyte is filled with lipid material that cell cytoplasm looks a black in microscope

[b]Oocyte is less filled with lipid material compared to black groups that the rim of cell cytoplasm appears white so it looks like white rings in microscope.

Table 2. Effects of insulin treatment on embryonic development of in vitro parthenogenetic pig oocytes

| | No. of oocytes develop to (%) | | |
|---|---|---|---|
| Treatment of insulin | Oocytes | Cleaved | Develop to blastocysts |
| Insulin (+) Black | 224 | 184(82.1±0.8) | 33(15.5±3.6) |
| Insulin (+) White ring | 136 | 86(63.2±0.3) | 16(11.7±1.3) |
| Insulin (-) Black | 185 | 145(78.3±0.1) | 30(16.6±0.4) |
| Insulin (-) White ring | 178 | 113(63.4±0.0) | 13(7.4±0.2) |

8 replicates were performed.

Table 3. Blastocysts cell number of porcine parthenogenetic oocytes after culture with insulin positive, insulin negative during in vitro maturation and culture

| Insulin treatment | Insulin (+) black | Insulin (+) white ring | Insulin (-) black | Insulin (-) white ring |
|---|---|---|---|---|
| Cell number | 33.2±2.5 | 31.2±2.1 | 35.5±2.6 | 31.3±2.2 |

8 replicates were performed.

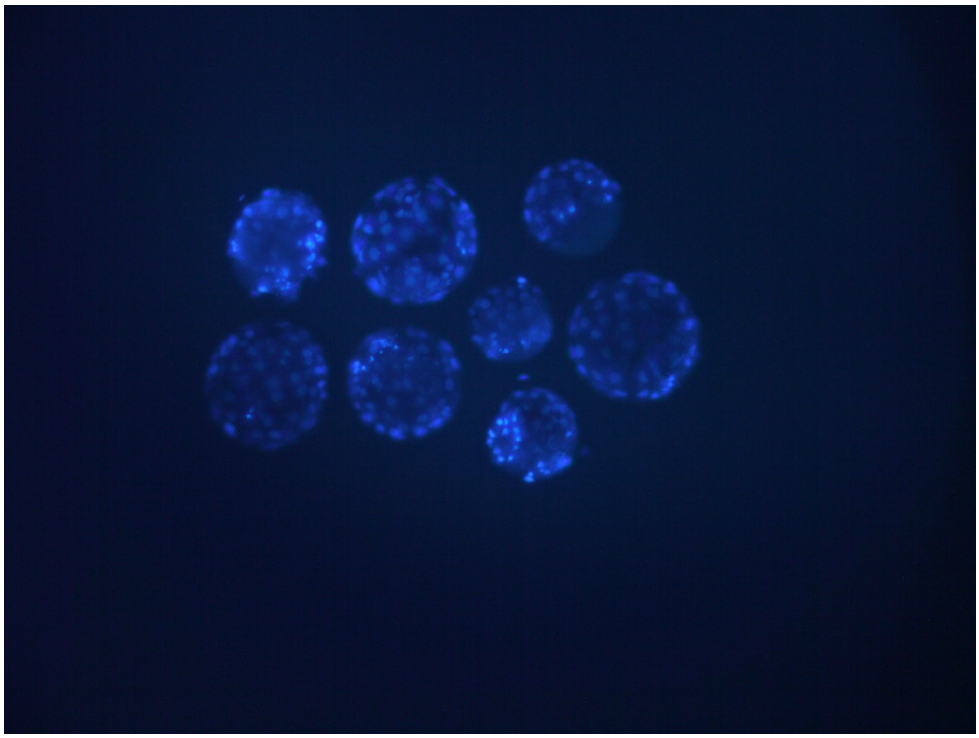

Fig. 3. At day 6 expanded blastocysts were fixed for total cell counting. Blastocysts were stained and counted total cell numbers.

groups, white ring groups. Table 3 shows that the cells in blastocyst was increased in the group with both insulin (+) and insulin (-) black groups (33.2% vs 31.2% and 35.5% vs 31.3%; respectively).

## DISCUSSION

The capability of porcine oocytes to *in vitro* mature, fertilize, and develop has been proved by several researchers, and successful methods for *in vitro* maturation and fertilization of porcine oocytes and culture of porcine embryos have been developed. But, even if pig oocytes develop to blastocyst stages by fertilization *in vitro* developmental potential is higher than *in vivo* (Day 1993). In the first experiment, determining of first polar body extraction oocytes were selected for in vitro maturation of parthenogenesis.

Growth factors such as insulin, IGF-1 have a regulatory effects on ovarian function (Hernandez et al 1988, Kolle et al 2003). In bovine, maturation of oocytes has pleiotropic effects on embryonic development in this species (Masui 2001, Nurse 1990, Taieb et al 1997). After insulin treatment and non-treatment, the oocytes were separated two groups; black groups that cell is filled with lipid material, cell cytoplasm looks a black in microscope, and white ring groups that cell is less filled with lipid material compared to black groups, the rim of cell cytoplasm looks white, so it looks like white rings in microscope. 8 replicated sampling were shown that cytoplasm with insulin positive oocytes was more black groups compared with the cytoplasm with insulin negative oocytes, for development and follicular survival insulin and FSH were more crucial in in vitro culture media (Hartshorne et al 1997). During growth and maturation within ovarian follicles some growth factors were important in mammalian oocytes such as epidermal growth factor (EGF), insulin like growth factor 1 (IGF-1), and insulin (van den Hurk & Zhao 2005). *In vitro* oocytes maturation, in pre-ovulatory follicles insulin was high when compared with subordinate follicles because of insulin effect (Landau et al 2000). Granulosa cells of ovarian proliferation and P4 production enhanced by insulin and steroidogenesis of luteal cells were increased in different species (Spicer & Echternkamp 1995).

In the second experiment, cleavages and blastocysts rates, black groups were significantly increased early development of cleavages rates and formation of blastocyst compared with white ring groups. Also, oocytes with insulin treatment showed higher development of embryonic development on rates of cleavage and formation of blastocysts compared with control

group. The capability of porcine oocytes to *in vitro* mature, fertilize, and develop has been proved by several researchers, and successful methods for *in vitro* maturation and fertilization of porcine oocytes and culture of porcine embryos have been developed. But, even if pig oocytes develop to blastocyst stages by fertilization *in vitro* developmental potential is higher than *in vivo* (Day 1993). Granulosa cells of ovarian proliferation and P4 production enhanced by insulin and steroidogenesis of luteal cells were increased in different species (Wilmut et al 1997). Because of that, rates of cleavage and formation of blastocyst rates were increased by insulin treatment.

There was significant difference in the total cell numbers of blastocyst. Total cell number of blastocyst is directly correlated with the quality of blastocyst. Total blastocyst cells number were significantly higher in the black groups with both positive and negative treatment of insulin than white ring groups.

In conclusion, within the research period clearly can identify the direct effect of insulin to embryonic maturation and development in the porcine oocytes. During IVM insulin can play role on development of embryos in different developmental stages. With insulin, cell cytoplasm with cell lipid material was filled more, and it makes black groups more. In the second and third experiment, blastocysts rates and cell numbers of blastocyst was increased in black groups with or without insulin. Insulin made cell cytoplasm good quality, so oocyte with good quality cell cytoplasm made more blastocyst cell number. To add insulin with IVM for embryo production and offspring in pigs can play beneficial effects.

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## REFERENCES

Adamiak SJ, Mackie K, Watt RG, Webb R, Sinclair KD. 2005. Impact of nutrition on oocyte quality: Cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. Biol Reprod 73: 918-26

Biswas D, Jung EM, Jeung EB, Hyun SH. 2011. Effects of vascular endothelial growth factor on porcine preimplantation embryos produced by in vitro fertilization and somatic cell nuclear transfer. Theriogenology 75: 256-67

Dadi TD, Li MW, Lloyd KCK. 2007. EGF and TGF-alpha supplementation enhances development of cloned mouse embryos. Cloning Stem Cells 9: 315-26

Day BA. 1993. Political Dissent and Napoleonic Representations during the Restoration Monarchy. Hist Reflections 19: 409-32

Freret S, Grimard B, Ponter AA, Joly C, Ponsart C, Humblot P. 2006. Reduction of body-weight gain enhances in vitro embryo production in overfed superovulated dairy heifers. Reproduction 131: 783-94

Gupta MK, Uhm SJ, Lee SH, Lee HT. 2008. Role of nonessential amino acids on porcine embryos produced by parthenogenesis or somatic cell nuclear transfer. Mol Reprod Dev 75: 588-97

Han ZM, Vassena R, Chi MMY, Potireddy S, Sutovsky M, et al. 2008. Role of glucose in cloned mouse embryo development. Am J Physiol-Endoc M 295: E798-E809

Hartshorne GM, Barlow A, Child TJ, Kennedy CR, Barlow DH, Hulten MA. 1997. Identification of meiotic cells in cultures of human fetal ovarian tissue. Hum Reprod 12: P177-P77

Hernandez ER, Resnick CE, Svoboda ME, Vanwyk JJ, Payne DW, Adashi EY. 1988. Somatomedin-C Insulin-Like Growth Factor-I as an Enhancer of Androgen Biosynthesis by Cultured Rat Ovarian-Cells. Endocrinology 122: 1603-12

Himaki T, Mizobe Y, Tsuda K, Suetomo M, Yamakuchi H, et al. 2012. Effect of Postactivation Treatment with Latrunculin A on In Vitro and In Vivo Development of Cloned Embryos Derived from Kidney Fibroblasts of an Aged Clawn Miniature Boar. J Reprod Develop 58: 398-403

Kolle S, Stojkovic M, Boie G, Wolf E, Sinowatz F. 2003. Growth hormone-related effects on apoptosis, mitosis, and expression of connexin 43 in bovine in vitro maturation cumulus-oocyte complexes. Biol Reprod 68: 1584-89

Landau S, Braw-Tal R, Kaim M, Bor A, Bruckental I. 2000. Preovulatory follicular status and diet affect the insulin and glucose content of follicles in high-yielding dairy cows. Anim Reprod Sci 64: 181-97

Lee GS, Kim HS, Hyun SH, Kim DY, Lee SH, et al. 2003. Improved developmental competence of cloned porcine embryos with different energy supplements and chemical activation. Mol Reprod Dev 66: 17-23

Lee SH, Kim DY, Nam DH, Hyun SH, Lee GS, et al. 2004.

Role of messenger RNA expression of platelet activating factor and its receptor in porcine in vitro-fertilized and cloned embryo development. Biol Reprod 71: 919-25

Lunney JK. 2007. Advances in swine biomedical model genomics. Int J Biol Sci 3: 179-84

Masui Y. 2001. From oocyte maturation to the in vitro cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CSF). Differentiation 69: 1-17

Nurse P. 1990. Universal Control Mechanism Regulating Onset of M-Phase. Nature 344: 503-08

ONeill C. 1997. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. Biol Reprod 56: 229-37

Pandey A, Gupta SC, Gupta N. 2009. EFFECT OF DIFFERENT CULTURE MEDIUM ON THE PRODUCTION OF GRANULOSA SCNT EMBRYOS IN WATER BUFFALO (BUBALUS BUBALIS) AND THEIR mRNA TRANSCRIPT ANALYSIS AT DEVELOPMENTAL STAGES IN COMPARISON WITH IN VITRO FERTILIZATION-PRODUCED EMBRYOS. Reprod Fert Develop 21: 194-95

Salahuddin S, Ookutsu S, Goto K, Nakanishi Y, Nagata Y. 1995. Effects of Embryo Density and Coculture of Unfertilized Oocytes on Embryonic-Development of in-Vitro Fertilized Mouse Embryos. Hum Reprod 10: 2382-85

Sjaastad I, Schiander I, Sjetnan A, Qvigstad E, Bokenes J, et al. 2003. Increased contribution of alpha(1) vs. beta-adrenoceptor-mediated inotropic response in rats with congestive heart failure. Acta Physiol Scand 177: 449-58

Song K, Hyun SH, Shin T, Lee E. 2009. Post-Activation Treatment With Demecolcine Improves Development of Somatic Cell Nuclear Transfer Embryos in Pigs by Modifying the Remodeling of Donor Nuclei. Mol Reprod Dev 76: 611-19

Spicer LJ, Echternkamp SE. 1995. The Ovarian Insulin and Insulin-Like Growth-Factor System with an Emphasis on Domestic-Animals. Domest Anim Endocrin 12: 223-45

Sugimura S, Yamanaka K, Kawahara M, Wakai T, Yokoo M, Sato E. 2010. Early metaphase II oocytes treated with dibutyryl cyclic adenosine monophosphate provide suitable recipient cytoplasm for the production of miniature pig somatic cell nuclear transfer embryos. Anim Sci J 81: 48-57

Taieb F, Thibier C, Jessus C. 1997. On cyclins, oocytes, and eggs. Mol Reprod Dev 48: 397-411

Thirthagiri E, Cheong LS, Yip CH, Teo SH. 2009. CHEK2*1100delC does not contribute to risk to breast cancer among Malay, Chinese and Indians in Malaysia. Fam Cancer 8: 355-58

van den Hurk R, Zhao H. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63: 1717-51

Vutyavanich T, Saeng-anan U, Sirisukkasem S, Piromlertamorn W. 2011. Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos. Fertil Steril 95: 1435-39

Wadhwa N, Kunj N, Tiwari S, Saraiya M, Majumdar SS. 2009. Optimization of embryo culture conditions for increasing efficiency of cloning in buffalo (Bubalus bubalis) and generation of transgenic embryos via cloning. Cloning Stem Cells 11: 387-95

Wakai T, Sugimura S, Yamanaka KI, Kawahara M, Sasada H, et al. 2008. Production of viable cloned miniature pig embryos using oocytes derived from domestic pig ovaries. Cloning Stem Cells 10: 249-61

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-13

Yamanaka K, Sugimura S, Wakai T, Kawahara M, Sato E. 2009. Difference in sensitivity to culture condition between in vitro fertilized and somatic cell nuclear transfer embryos in pigs. J Reprod Dev 55: 299-304

Yamanaka K, Sugimura S, Wakai T, Shoji T, Kobayashi J, et al. 2007. Effect of activation treatments on actin filament distribution and in vitro development of miniature pig somatic cell nuclear transfer embryos. J Reprod Dev 53: 791-800

Yoshioka K. 2011. Development and Application of a Chemically Defined Medium for the In Vitro Production of Porcine Embryos. J Reprod Develop 57: 9-16

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