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Association between HSP70 Genotypes and Oocytes Development on *In vitro* Maturation/Fertilization in Pig

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ABSTRACT : This study was performed to clarify whether the variation of stress related heat shock protein 70 (HSP70) (GenBank X68213) gene was associated with the nuclear morphological change of *in vitro* maturation and *in vitro* capacitation in oocytes of pig ovaries obtained at the slaughterhouse. The nucleic acid substitution of C to G at the 483rd position was found out in HSP70 K1 (290-512) from X68213. The ovaries were categorized into CC, CG, and GG genotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (BsiHKA I). After the second *in vitro* maturation of immature fresh oocytes, the relation of nuclear morphological change in oocytes with the genotype of HSP70 K1 gene was such that the MII ratios of the genotype GG and CG (46.93% and 42.20%, respectively) were significantly higher than that of the CC genotype (10.71%) ($p<0.05$). With respect to *in vitro* maturation of frozen-thawed oocytes by an open pulled straw (OPS) method, the percentage of oocytes matured to MII stage of the CG genotype showed a higher trend than CC and GG genotypes. After the *in vitro* maturation of immature fresh oocytes and frozen-thawed oocytes by the OPS method, the relation of the pronuclei change in oocytes matured *in vitro* with HSP70 genotype was assessed, and the result showed that the enlarged sperm heads (ESH) of matured fresh oocytes and frozen-thawed oocytes were 80.0% and 60.0% in the CC genotype, respectively. The CC genotype group had a significantly higher rate of ESH than the CG and the GG genotype group ($p<0.05$). The ratios of polyspermic invasion were not different among HSP70 of the three genotypes. It was considered that the rate of *in vitro* maturation of fertilized oocytes was expected to differ according to genotype of the stress related gene. (**Key Words** : Fertilization, Maturation, Porcine Oocytes, HSP70 Genotypes)

INTRODUCTION

Porcine HSP reacts on cell growth substantially under stressful conditions caused by the change of temperature, particularly, in response to sudden elevation of the external temperature, HSP is very important for the maintenance of the cell viability of fetus or neonates (Dezeure et al., 1993; Lindquist, 1986). Although its importance on the protection of reproductive cells from temperature stress has not been elucidated yet, several HSP gene groups have been reported to mediate great effects on the development of normal reproductive cells (Allen et al., 1988; Zakeri et al., 1990). HSP70 gene is involved in proteins secreted by rising and dropping temperature, and pigs which were inherited DNA variation of physiological in adaptability to temperature changes are speculated to cause the deterioration of the productibility and breeding capacity of pigs (Huang et al., 2000, 2002). In addition, HSP70 that is a group of proteins expressed in response to stress in pigs has the function of heat tolerance and immune reaction. HSP70 gene is located on chromosome 7, and it is a protein classified as porcine major histocompatibility complex (MHC) class III group (Rothschild and Ruvinsky, 1998).

If oocytes are directly exposed to high heat during *in vitro* maturation, the phenomenon of nuclear maturation disrupts spindle formation at the metaphase I in mice (Baumgarner and Chrisman, 1981; Davis, 1985), and reduces the progress to metaphase II and the fertilization rate in mice and cattle (Baumgartner and Chrisman, 1981; Lenz et al., 1983). In addition, abnormalities of that chromosome and cytoplasm could decrease matured oocytes in porcine. This phenomenon mediates an important effect on the development of fertilized oocytes caused by

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the change of the temperature (Ju and Tseng, 2004). Particularly, porcine HSP70 has been reported to be a key molecule for the viability of oocytes during *in vitro* maturation of follicular oocytes (Neuer et al., 1999) and the protection of cells from heat shock stress (Ananthan et al., 1986; Lindquist, 1986; Welch, 1992; Hansen, 1999). Hendrey and Kola (1991) have reported that injection of a small amount of HSP70 mRNA showed the increase of the resistance from heat shock in rat oocytes. During the development of *in vivo* and *in vitro* fertilized oocytes, elevation of temperature may cause the impairment of the expression of specific genes and protein synthesis in cytoplasm at the molecular level within the cell, and the embryo development rate may be decreased. In addition, it is speculated that heat shock stress effects on the early embryo directly, and it also influences the development to the late embryo (Alliston et al., 1965; Ealy et al., 1993; Edwards and Hansen, 1997). The failure of heat shock protein induction after receiving heat shock also could be observed in bovine oocytes, and immature bovine oocytes could exhibit the reaction of heat shock cognate (HSC70) and HSP70 induction associated protein, nonetheless, the reaction rate of between these two proteins are not changed by heat shock (Edwards and Hansen, 1996). King et al. (2000) have reported that after the blocking stage, the necessity of HSP60 and HSP72 protein were increased for the development of porcine fertilized oocytes. This phenomenon proved that the HSP gene group is essential for the development of early porcine embryos. During the *in vitro* maturation of porcine follicular oocytes, the heat reaction of HSP70 gene functioned as an important factor of the *in vitro* fertilization and development (Fukui, 1991). However, Kawarsky and King (2001) have reported that the induction of HSP70 was not influenced by the exposure to high temperature in immature or mature oocytes, and this protein was closely associated with spindles formation during meiosis. Thus, it mediates an effect of the stability of the structure of bovine cytoplasmic micro-organisms (Kawarsky and King, 2001).

This study was examined whether the variation of stress related HSP70 gene was associated with nuclear morphological change during *in vitro* maturation with or without freezing of immature oocytes on the pattern of the enlarged sperm head and polyspermic invasion in pigs.

MATERIALS AND METHODS

In vitro maturation and fertilization of follicular oocytes

The ovary was obtained from female pigs killed at a slaughterhouse, and immersed in sterilized saline (30-34°C), from a visible follicle 2-6 mm in diameter. Immature oocytes were collected by aspiration follicular fluid with a syringe attached an 18 gauge needle. Only dense oocytes and even cytoplasms were selected under stereoscopic microscope. The first maturation culture was performed with NCSU23 maturation medium (Petter et al., 1993) added 10% porcine follicle fluid (PFF) and hormones (FSH 0.5 μg/ml, LH 0.5 μg/ml, 10 ng/ml EGF, 50 μm β-mercaptoethanol, and 10 IU/ml hCG; Sigma) at 5% CO_2, under the high humidity gas condition in 38.5°C for 24 h. The second culture was carried out with NCSU23 culture medium without adding hormones for 22 h. *In vitro* fertilization was prepared by thawing frozen sperm straws (0.5 ml) in 37°C water bath for 30 seconds, washing two times with mTBM (Abeydeera and Day, 1997) containing bovine serum albumin (1 mg/ml, bovine serum albumin, BSA; Sigma) by centrifuging (900×g, 5 minutes), and diluting with mTBM added 2 mM caffeine (Sigma, USA) to 1.5×10^5/ml sperm concentration. Follicular oocytes matured *in vitro* for 44-46 h were selected, and the cumulus cells were removed in maturation culture medium containing 0.1% hyaluronidase (Sigma, USA) by repeat pipetting methods. *In vitro* fertilization was carried out for 6 h with mTBM fertilization medium in the 38.5°C, 5% CO_2 incubator.

OPS freezing and thawing

For the open pulled straw (OPS) freezing method, the freezing process reported by Vajta et al. (1997) was modified and used. The holding medium (HM) for freezing was used to be TCM-199 (Gibco, Life Technologes INC. USA) added 2.5 mM Hepes and 20% FCS basically. The sucrose media was used to be TCM-199 added 0.6 M sucrose and 20% fetal calf serum (FCS, Gibco). In the composition of freezing medium used for freezing, vitrification solution (VS) 1 were used to HM with 10% Ethylene Glycol (EG) and 10% dimethyl sulfoxide (DMSO), and VS 2 were used to sucrose media with 20% EG and 20% DMSO. As concerning of the freezing process, HM and VS1 were added, covered with mineral oil, and pre-incubation was carried out for 2-3 h in a 4 well dish prior to experiments. The OPS were used to capillary tubes (Sigma, USA) of 25 μm diameter and 200 μl cryoprotector. On the other hand, oocytes were washed by transferring to HM, equilibrated in VS1 solution for 3 minutes, and exposed in VS2 solution with cryoprotector for 30 seconds. Immediately after the procedure, freezing was carried out by adding 5 oocytes to VS2 drop solution of 2 μl, aspirating oocytes by capillary reaction, and depositing within liquid nitrogen(LN_2, -196°C) immediately. Frozen straws were transferred to a LN_2 container, and were stored more than 7 days. As the thawing media, HM and 0.6 M sucrose solution were mixed at 2:1 ratio and used as the thawing medium (TM) 1, and as TM2, HM and 0.6 M sucrose were mixed at 4:1. Immediately after taking out straws from the LN_2 container, the straws were thawed by adding to TM1

media for 5 minutes, maintained in TM2 for 5 minutes, and then in HM for 5 minutes.

Analysis of the nuclear morphology of oocytes

Cumulus cells which were attached at oocytes after the second *in vitro* maturation and oocytes after 24 h of insemination were removed by physical removal method (Lu et al., 1987; Cox, 1991) using micro pasture pipette in mTBM solution with 0.2% hyaluronidase (Xu et al., 1987; Park et al., 1989). Nuclear morphology was examined according to the method reported by Monaghan et al. (1993). After the removal of cumulus cells, the oocytes were fixed in the fixation solution of 1:3 (acetic acid:methanol) for approximately for 2 minutes, and were stained with 1% orcein (Sigma, USA), and were fixed during the 2 days in acetic alcohol. Then the nuclear type was observed under light microscope. The change of the nucleus type of immature fresh oocytes and frozen-thawed oocytes was examined at first and in regard to the evaluation of the nucleus type, oocytes without the loss of nucleus membrane was defined as germinal vesicles (GV). The oocytes of which GV was destroyed and entered the migration phase was determined to be the prophase-I (PI) to telephase-I (TI), and the metaphase - II (MII) (McGaughey and Polge, 1971). After the *in vitro* fertilization of immature oocytes and frozen-thawed oocytes, the pronuclei of the fertilized oocytes were observed. *In vitro* fertilized oocytes were also analyzed according to the method reported by Monaghan et al. (1993). *In vitro* fertilized oocytes were stained for 6 h as method confirming fertilized oocytes, and observed nuclear morphology of enlarged sperm head (ESH), both pronuclei (BPN; male pronuclei, female pronuclei) and polyspermic invasion were observed separately.

Genomic DNA separation and genotype analysis

Extraction of genomic DNA from porcine ovarian tissues was performed by partially modifying the method report by Miller et al. (1988).

To amplify the HSP70 gene on *in vitro* maturation of porcine oocytes, Forward 5'-CCCTGAATCCGCAGAA TACC-3' and Reverse 5'-TACGCCTCCGCAGTCTCCTT-3' HSP70 K1 primers reported by Jin et al. (2005; 2006) were used. For PCR reaction, approximately 50-80 ng template DNA, 10 pmol each primer, 50 μM each dNTP, 10× reaction buffer, and 1 unit Taq polymerase (Bioneer Co., Korea) were mixed, and the final volume was adjusted to 25 μl. As the reaction condition, using a PCR system (Gene Amp. 9700, Perkin Elmer, USA), pre-denaturation at 94°C for 5 minutes, and 35 cycles of reactions 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 30 seconds were performed, and the final-extention was carried out at 72°C for 5 minutes.

To perform single-strand conformation polymorphism

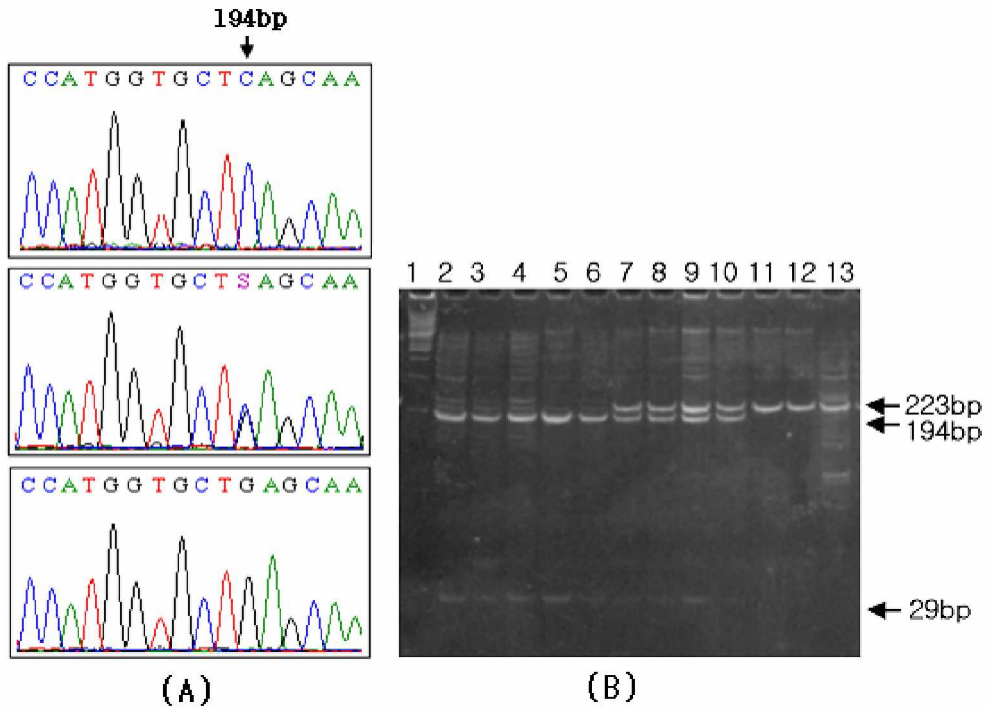

Figure 1. (A) Partial electrophotograms of HSP70 K1 sequence of HSP70 gene (the upper part: CC homozygous, the middle part: CG heterozygous, the lower part: GG homozygous). (B) PCR-RFLP allelic patterns of HSP70 K1 gene obtained by digestion with BsiHKA I on 15% polyacrylamide gel (1 lane; 100 bp marker, 7-10 lane; CG (223 bp, 194 bp, 29 bp), 11,12 lane; GG (223 bp), 2-6 lane; CC (194 bp, 29 bp), 13 lane; PCR product).

Table 1. Effects of HSP70 K1 gene on morphological changes of nuclear during *in vitro* maturation in porcine fresh oocytes

Genotype	No. of oocytes cultured	No. (%) of oocytes at stage of			No. (%) of oocytes non-examined
		GV	PI~TI	M II	
CC	28	2 (7.14)	9 (32.14)	3 (10.71)[b]	14 (50.00)[a]
CG	1,261	190 (15.07)	441 (34.97)	570 (45.20)[a]	60 (4.76)[b]
GG	2,363	352 (14.90)	829 (35.08)	1,109 (46.93)[a]	73 (3.09)[b]

GV = Germinal vesicle, PI = Prophase I, TI = Telephase I, M II = Metaphase II.
[a, b] Values with different superscripts within columns are significantly differ ($p<0.05$).

Table 2. Effects of HSP70 K1 gene on morphological changes of nuclear during *in vitro* maturation of porcine immature oocytes frozen-thawed by open pulled straw (OPS) methods

Genotype	No. of oocytes cultured	No. (%) of oocytes at stage of			No. (%) of oocytes non-examined
		GV	PI~TI	MII	
CC	27	16 (59.26)	7 (25.93)	0 (0.00)	4 (14.81)
CG	1,350	551 (40.81)	588 (43.56)	151 (11.19)	60 (14.44)
GG	353	129 (36.54)	173 (49.00)	21 (5.95)	30 (8.50)

GV = Germinal vesicle, PI = Prophase I, TI = Telephase I, MII = Metaphase II.

(SSCP), PCR amplification products were denatured at 94°C for 10 minutes, and cooled immediately on ice for 5 minutes. The genetic polymorphism of denatured DNA samples was examined by 30% MDE gel (Cambrex, USA). Products of which genetic polymorphism was confirmed were purified with ethanol, and cycle sequence was performed by the use of the Bigdye terminator kit ver.3.0 (Applied Biosystems, USA). The reaction condition was 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Nucleic acid sequencing was performed by using the ABI3130 instrument (Applied Biosystem, USA). To detection of PCR-RFLP genotype, amplified PCR products were incubated with restriction enzyme BsiHKA I (NEB, USA) recognizing 5'-GTGCT▼C-3' and 5'-GTGCTG-3' at 65°C for 2 h in a constant temperature water bath, and the polymorphism of loading incubated PCR products gel were assessed after the electrophoresis on 15% polyacrylamide gel.

Statistical analysis

The effect of HSP70 K1 genotype on the change of nucleus types of *in vitro* maturation as well as *in vitro* fertilization was analyzed by general linear model (GLM) using the statistics analysis system (SAS) software package (1999). The least squares mean (LSM) of each factor was estimated, and a significance of the difference of the mean of each factor was validated by least squares difference (LSD) test for the multiful comparison.

RESULTS AND DISCUSSION

In nucleic acid sequence 483rd of porcine HSP70 gene region, the substitution of C to G was confirmed by nucleic acid analysis (Figure 1A), and the RFLP polymorphisms of the restriction enzyme BsiHKA I is shown in Figure 1(B). The C allelic gene was restricted to 194 bp and 29 bp fragments, on the other hand, the G allelic gene was expressed as 223 bp states without digestion. Therefore, oocytes with the C allelic gene was the pattern of bands with 194 bp and 29 bp only, and the heterozygote of C and G allelic gene showed the pattern with 223 bp, 194 bp and 29 bp bands, and the G allelic gene homozygote showed the pattern of 223 bp band. After the second *in vitro* maturation, nucleus morphology of oocytes with the PCR-RFLP genotype of HSP70 K1 region were showed the percentages reaching the M II that the CC genotype was 10.71%, and the GG genotype and the CG genotype were 46.93% and 45.20%, respectively. The GG and CG genotype groups showed the significantly higher result than the CC genotype group ($p<0.05$) (Table 1). Fukui et al. (1991) have reported that the maturation condition of the nucleus and cytoplasm of follicular oocytes plays a decisive role in the growth of *in vitro* fertilized oocytes after fertilization, and cumulus cells mediate an important effect on acrosomal reaction of sperms, *in vitro* fertilization and the growth of fertilized oocytes in pigs.

Edward et al. (1996) were reported for the *in vitro* maturation of immature follicular oocytes that the nucleus of oocytes arrested at the prophase-I of the first maturation division reinitiated maturation division and reached the metaphase of the second meiosis by performing *in vitro* culture for 44-46 h. However, the change of nucleus of oocytes did not show a significant difference among three genotypes in frozen-thawed oocytes by OPS method. Nonetheless, it was found that the CG genotype showed a slightly higher trend than the other two groups in concerning the rate of the maturation to the optimal fertilization stage of the MII stage (Table 2). These results are similar to the findings reported by Huang et al. (2000, 2002) that HSP70 gene was involved in protein secretion of pigs *in vivo* response to the change of temperature to high temperature as well as low temperature, and physical responsibility of the changing temperature was lowered in pigs with inherited DNA variation factors.

Table 3. Effects of HSP70 K1 gene on *in vitro* fertilization in porcine fresh oocytes

Genotype	No. of oocytes inseminated	No. (%) of oocytes fertilized with			No. (%) of polyspermic oocytes	No. (%) of unfertilized oocytes
		Total	ESH	BPN		
CC	100	84 (84.00)[a]	80 (80.00)[a]	4 (4.00)	0 (0.00)	16 (16.00)[b]
CG	365	221 (60.55)[b]	174 (47.67)[b]	47 (12.88)	25 (6.85)	119 (32.60)[a]
GG	1,680	971 (57.79)[b]	706 (42.02)[b]	265 (15.77)	139 (8.27)	570 (33.94)[a]

ESH = Enlarged sperm head, BPN = Both pronuclei.
[a, b] Values with different superscripts within columns are significantly differ ($p<0.05$).

Table 4. Effects of HSP70 K1 gene on *in vitro* fertilization of porcine immature oocytes frozen-thawed by open pulled straw (OPS) method

Genotype	No. of oocytes inseminated	No. (%) of oocytes fertilized with			No. (%) of polyspermic oocytes	No. (%) of unfertilized oocytes
		Total	ESH	BPN		
CC	50	30 (60.00)[a]	30 (60.00)[a]	0 (0.00)	0 (0.00)	20 (40.00)[b]
CG	51	17 (33.33)[b]	16 (31.37)[b]	1 (1.96)	3 (5.88)	31 (60.79)[a]
GG	157	53 (33.76)[b]	45 (28.66)[b]	8 (5.10)	12 (7.64)	92 (58.60)[a]

ESH = Enlarged sperm head, BPN = Both pronuclei.
[a, b] Values with different superscripts within columns are significantly differ ($p<0.05$).

The results are related to examination among pronuclei of *in vitro* fertilized oocytes, which are fresh and frozen-thawed oocytes, and the PCR-RFLP polymorphism of HSP70 K1 gene were presented in Table 3 and 4. Three genotypes in Table 3 are compared to the results of ESH and BPN *in vitro* fertilized oocytes from fresh oocytes. The sperm pattern and the presence or absences of pronuclei were examined at 24 h after fertilization with oocytes matured *in vitro*. The CC genotype group is estimated to 80% of the ESH formation rate and 4% of BPN formation rate in 100 oocytes inseminated. The CG genotype group is estimated to 47.67% and 12.88% of the ESH and BPN formation rate in inseminating 365 oocytes, respectively. The GG genotype group showed the results of 42.02% and 15.77% of the ESH and BPN formation rate in 1,680 oocytes inseminated. Among the entire oocytes used, the CC genotype group showed significantly higher rate of ESH formation than CG and GG genotypes. But the rate of BPN formation was not showed difference among three genotypes. Jin et al. (2005) have reported the study on the association between SSCP genotype of HSP70 K1 from ovary DNA and early development of *in vitro* fertilized oocytes that the rate of cleaved oocytes of the AA type and the AB type were significantly different by the genotypes ($p<0.05$). Those results coincide with this study that ESH was significantly higher in the CC genotype. Immature oocytes were thawed after vitrification frozen by the OPS method and fertilized after *in vitro* mature for 48 h (Table 4). The rate of the formation of ESH and BPN were examined in 6 hours after fertilization. The total *in vitro* fertilization rate of 60% for CC genotype group was showed significantly higher fertility than that of 33.33% and 33.76% for CG and GG genotype groups in 50 oocytes ($p<0.05$). Concerning the rate of the ESH formation, the CC genotype group of 60.00% was significantly than the CG and GG genotype groups of 31.37% and 28.66%, respectively ($p<0.05$). However, the rate of the BPN formation for 0%-7.64% level did not show significant difference among genotype groups Reviewing the result of Table 3 and 4, the CC genotype allelic groups of both fresh and frozen-thawed oocytes showed a significantly higher rate on regard to the rate of the ESH formation ($p<0.05$). However, in the rate of polyspermic invasion, the CG and the GG genotype groups showed a slightly higher rate than the CC genotype group. These showed the similar results reported by Ball et al. (1983) and Nagai (1994) that porcine oocytes were different from the other animal's oocytes occurred incomplete fertilization by high polyspermic invasion and low rate of pronuclei formation during *in vitro* fertilization.

Important factors determining the success of porcine *in vitro* fertilization are maturity of follicular oocytes, sperm activity and concentration, and temperature changes (Mattioli et al., 1991; Kikuchi et al., 1993). Allen et al. (1988) and Zakeri et al. (1990) have reported that several HSP genes mediate great effects on the development of normal reproductive cells. Neuer et al. (1999) have reported that high sensitivity of oocytes was associated with the reaction of HSP70 gene express during the meiosis maturation, and it is the key molecules for the protection of cells from heat shock and stress (Ananthan et al., 1986; Lindquist, 1986; Welch, 1992; Hansen, 1999). HSP 70 gene is a defense mechanism against external heat stress, and if the expression regulation process or pertinent genes were elucidated, it is expected that the low productivity may be prevented in association with reproductive ability. According to the report of Fukui et al. (1991), in bovine cases, the maturation condition of nucleus and cytoplasm of follicular oocytes play a decisive role in the development of *in vitro* fertilized oocytes after fertilization. In addition, it was considered that the rate of *in vitro* maturation and fertilized oocytes were different according to genotype of

stress relating gene. In the result of this, the rate of *in vitro* maturation oocytes of fresh oocytes up to the metaphase II estimated significantly high in the GG genotype group ($p<0.05$). But the rates of ESH formation after *in vitro* fertilization from fresh and frozen-thawed oocytes were estimated significantly high in the CC genotype group ($p<0.05$). It is considered that more subsequent studies are required for the association of the *in vitro* fertilized embryo development and stress-relating gene from these results.

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