Effect of Vitrification on *In Vitro* Maturation and Development and Gene Expression in Canine Oocytes

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ABSTRACT

The *in vitro* maturation rate of vitrified-thawed canine oocytes was 30.8±3.4%. The *in vitro* maturation rate of vitrified oocytes was lower than that of the control (52.0±2.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes were significantly ($p<0.05$) lower than those of fresh oocytes. The *in vitro* maturation and developmental rates of the vitrified-thawed oocytes were 17.5±2.5% and 8.8±3.4%, respectively. This results were lower than the control group (43.6±3.2% vs 20.0±3.0%). SOD1 gene expression of 1~2 mm of follilce size were higher than those of above 6 mm follicle size. SOD2 gene expression of 1~2 mm of follicle size were significantly higher than those of above 6 mm follicle size ($p<0.01$). The expression pattern of SOD1, 2 was constantly expressed in both groups but strongly expressed in follicles (1~2 mm) group when compared to the above 6 mm follicles. SOD gene expression between groups the fresh and vitrified oocytes groups were significant differences in rates. However, RGS gene expression between groups the fresh and vitrified oocytes groups were no significant differences in rates.

(Key words : Canine oocytes, *In vitro* development, Vitrification, Gene expression)

INTRODUCTION

The small pet dog's reproduction is mainly completed by unconfirmed male dog of natural copulation and the proliferation of bad gene was worried. Embryonic development of canine oocytes that were matured and fertilized *in vitro* was recently achieved. The rates of cleavage are reported 8% and 37% in culture (Bedford *et. al*, 2003, Songsasen *et al.*, 2002, 2005; Rodrigues *et al.*, 2004; Otoi *et al.*, 2005). The low rates of canine embryonic development are a testament to the inefficient development of oocytes matured *in vitro*. Vitrification of canine oocytes with ethyleneglycol, dimethylsulfoxide (DMSO) and trehalose supplemented with trehalose reduces cell toxicity, detrimental temperature and cell damage but also prevents excess penetration to increase survival rates (Clark *et al.*, 1984; Fahy *et al.*, 1984; Sutton, 1982; Utsumi *et al.*, 1982). Turathum *et al.* (2010) apoptosis-related genes (GADPH and SOD) are more strongly expressed in vitrified-thawed cells when compared to control. The G protein-coupled receptor (GPCR) is ubiquitously found throughout the body. It is classically composed of seven transmembrane spanning receptors, heterotrimeric G proteins, and effectors (Berman and Gilman, 1998; Ross and Wilkie, 2000). Regulators of G protein signaling (RGS) proteins have recently been identified in mammals as negative RGS pathways, which consist of the four components of the GPCR system (Srinivasa *et al.*, 1998; Watson *et al.*, 1996). Although the presence of some RGS proteins in platelets has been reported (Martin *et al.*, 2001; Gagnon *et al.*, 2002; Garcia *et al.*, 2004), little is known about expression in relation to known RGS genes.

Therefore, the purpose of this study was to investigate the effects of vitrification on nuclear maturation and SOD and RGS gene expression patterns vitrified-thawed immature canine oocytes.

MATERIALS AND METHODS

In Vitro Maturation of Oocytes

Each experiment was replicated four times. Canine oocytes collected from slaughter house-derived ovaries were cultured *in vitro* for 40~48 h in TCM-199 (Whittaker, U.S.A.) medium supplemented with 10% FCS, 1 μg/ml of FSH, 2 IU/ml of hCG, 1 μg/ml of β-estradiol, 100 IU/ml of penicillin G and 100 μg/ml of streptomycin sulfate. Oocytes were aspirated from medium size follicles with an 18 gauge fixed to a 10 ml disposable syringe. The cumulus-oocytes complexes (COCs) that had an evenly distributed cytoplasm and washed three times in oocyte maturation medium containing hormonal

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supplements. Then each group of 50 COCs was cultured in 500 μl of maturation medium, which had previously been covered with mineral oil. Recovered COCs were cultured with TCM-199 medium supplemented with 10% (v/v) FCS (fetal calf serum) and 1 mg/ml of cysteine, 20 ng/ml of E_2, 10 IU/ml of HCG, 10 IU/ml of PMSG and 10 ug/ml of gonadotropin. Oocytes were transferred to 50 ul drops of maturation medium covered mineral oil and cultured in a CO_2 incubator (5% CO_2, 95% air, 38℃). Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical (St Louis, MO, USA). Oocytes were fixed in acetic acid : ethanol (1:3) solution for 24 h then stained using with 1% acetoorcein or 10 μg/ml bisbenzimide (Hoechst 33342) and observed under an fluorescence microscope (Nikon, Japan). The judgement of oocytes maturation *in vitro* was carried out depending on the criteria of maturation by cell and nuclear division, and *in vitro* development by investigating oocytes of development *in vitro*.

Vitrification and Thawing of Oocytes

Before dehydration, the COCs were treated with 3.0 μg cytoclacin B for 30 min at 38℃. The oocytes were transferred into a 1 ml cryotube containing 5 ul of 1 M DMSO at 25±2℃. Subsequently, 95 ul of EDS (20% EG+ 20% G+0.3 M sucrose) in 10% FCS+TCM-199 medium at 0℃, were added to each cryotube. After the cryotubes had been placed in ice water for 5 min, the oocytes were sealed in a 1.0 mm straw and they were plunged directly into LN_2 container and stored until use. Frozen oocytes were rapidly thawed in a water bath at 30~35℃, and then placed in TCM-199 medium containing 0.5 M sucrose for 5 min each, respectively, at 38℃. After being washed for 2~3 times, using fresh medium the oocytes were cultured in a 10% FCS+TCM-199 medium, The COCs were transferred to a 100 μl droplet of the maturation medium under mineral oil in a petri dish and cultured at 38℃ under 5%.

Gene Expression of Vitrified-Warmed Canine Oocytes

They were collected at 0 and 48 h of *in vitro* maturation to investigate gene expression using RT-PCR. All transcripts of COCs were performed by PCR using the Array Pure Nano-scale Purification Kit (Epicentre, WI, USA). The examined genes were apoptosis related superoxide dismutase (SOD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Regulators of G protein signalling (RGS4). Oocytes transferred to a 15 ml test tube containing 1 ml of a citrate phosphate dextrose solution (90 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, 14 mM $C_6H_8O_7 \cdot H_2O$, 128.7 mM $NaH_2PO_4 \cdot H_2O$, 2.55 g/100 ml dextrose). Samples was centrifuged at 1,000 rpm for 7 min to get platelet-rich plasma. Semiquantitative RT reaction procedures were performed using RT premix (Bioneer Co., Korea). Briefly, 2 mg of RNA were incubated with oligo dT18 at 0℃ for 5 min and cooled on ice for 3 min. After the addition of RT premix, there action mixture was incubated at 42.5℃ for 90 min, and there action was terminated by heating the samples at 95℃ for 5 min. The PCR reaction was performed using the PCR premix (Bioneer Co, Korea) with the appropriate sense and antisense primers under the following incubation conditions : a 45s denaturation at 94℃, an annealing of 45s at 55~60℃, an extension of 45 sat 72℃, and a final extension of 10 min at 72℃ at the end of the cycles. The PCR products were separated in a 1% agarose gel. The amplified PCR products were subjected to electrophoresis for visualization on a 1.5% agarose gel follow by UV analysis using the BioDoc-It System (UVP, Upland, CA, USA). The primers used for RT-PCR analysis are shown in Table 1.

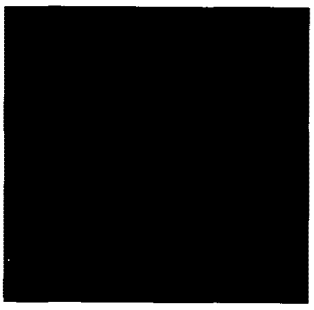

Fig. 1. Morphological appearance of oocytes after vitrification were stained with PI.

Table 1 Primers used for RT-PCR analysis

Gene	primers	Sequence (5'-3')	Tm (℃)
SODs	Forward	AGTGGGCCTGTTGTGGTATC	62.4
	Reverse	AGTCACATTGCCCAGGTCTC	62.4
RGS	Foward	GAAGATACGTTTTTGTGCCAGGTC	72.0
	Reverse	CTTCTGGATCAGCTGTGAGGA	72.0

Statistical Analysis

All data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the statistical analysis system (SAS Institute, Gary, NC, USA). Differences among treatment means were determined using Duncan's multiple range test and *t*-test. All the data were expressed as least square mean±S.D. Differences among treatment effects were considered at $p<0.05$).

RESULTS

Effects of Vitrification on *In Vitro* Maturation of Canine Oocytes

Table 2. Effects of vitrification on *in vitro* maturation of immature canine oocytes

Treatment	No. of oocytes examined	No. of oocytes matured to (%)	
		GV	MII
Control	100	44.0±3.2	52.0±2.5[a]
Vitrification	325	27.1±3.8	30.8±3.4[b]

[a,b] Values within column with different superscript differ ($p<0.05$).

Table 3. *In vitro* development of vitrified-thawed canine oocytes

Treatment	No. of oocytes examined	No. of oocytes matured (%)	No. of oocytes developed (%)
Control	110	43.6±3.2	20.0±3.0[a]
Vitrification	312	17.5±2.5	8.8±3.4[b]

[a,b] Values within column with different superscript differ ($p<0.05$).

The *in vitro* maturation rate of vitrified-thawed canine oocytes are shown in Table 2.

The *in vitro* maturation rate of vitrified-thawed oocytes was 30.8±3.4%. The *in vitro* maturation rate of vitrified oocytes was lower than that of the control (52.0± 2.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes were significantly ($p<0.05$) lower than those of fresh oocytes.

In Vitro and Development of Vitrified-thawed Oocytes

In vitro maturation and developmental rates of vitrified-thawed canine oocytes without distinct differences compared to fresh oocytes are shown in Table 3.

The *in vitro* maturation rates of the vitrified-thawed oocytes were 17.5±2.5%. This results were lower than the control group (43.6±3.2%). The *in vitro* developmental rates of the vitrified-thawed oocytes were 8.8±3.4%. This results were lower than that of the control group (20.0± 3.0%).

SOD Gene Expression of Follicle Size

SOD gene expression depending on follicle size are shown in Fig. 2. SOD1 gene expression of 1~2 mm of follicle size were higher than those of above 6 mm follicle size. SOD2 gene expression of 1~2 mm of follicle size were significantly higher than those of above 6 mm follicle size ($p<0.01$). The expression pattern of SOD-1, 2 was constantly expressed in both groups but strongly expressed in follicles (1~2 mm) group when compared to the above 6 mm follicles.

Gene Expression of Fresh and Vitrified Canine Oocytes

Gene expression of the fresh and following vitrification to investigate using RT-PCR. In order to assess the effect of vitrification on the expression of SOD and RGS,

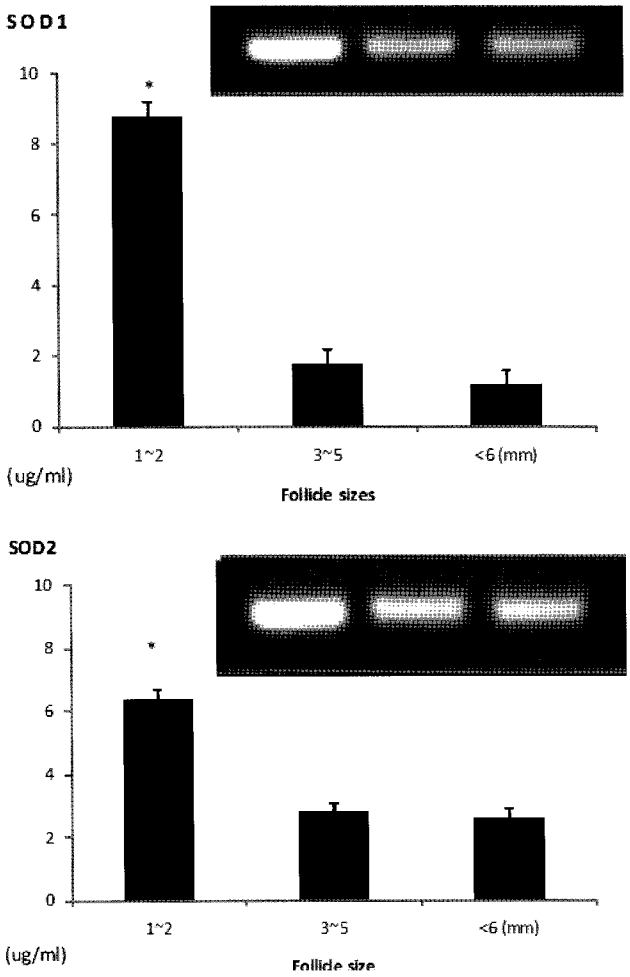

Fig. 2. Expression of SOD1 (up) and SOD2 (down) proteins in follicular fluids from small (1~2 mm), medium (3~5 mm), and large (> 6 mm) antral follicles.

the expression pattern of these selected genes in fresh and vitrified oocytes were analyzed (Fig. 3). SOD gene expression between groups the fresh and vitrified oocytes groups were significant differences in rates. However, RGS gene expression between groups the fresh and vitrified oocytes groups were no significant differences in rates. The expression pattern of SOD and RGS did differ between fresh (control) and vitrified oocytes. For SOD gene was constantly expressed in both groups but strongly expressed in vitrified-thawed group when compared to the control. RGS gene was no differences in rates vitrified-thawed group when compared to the control.

DISCUSSION

Recently, the *in vitro* maturation and fertilization of canine oocytes was achieved *in vitro*. The rates of clea-

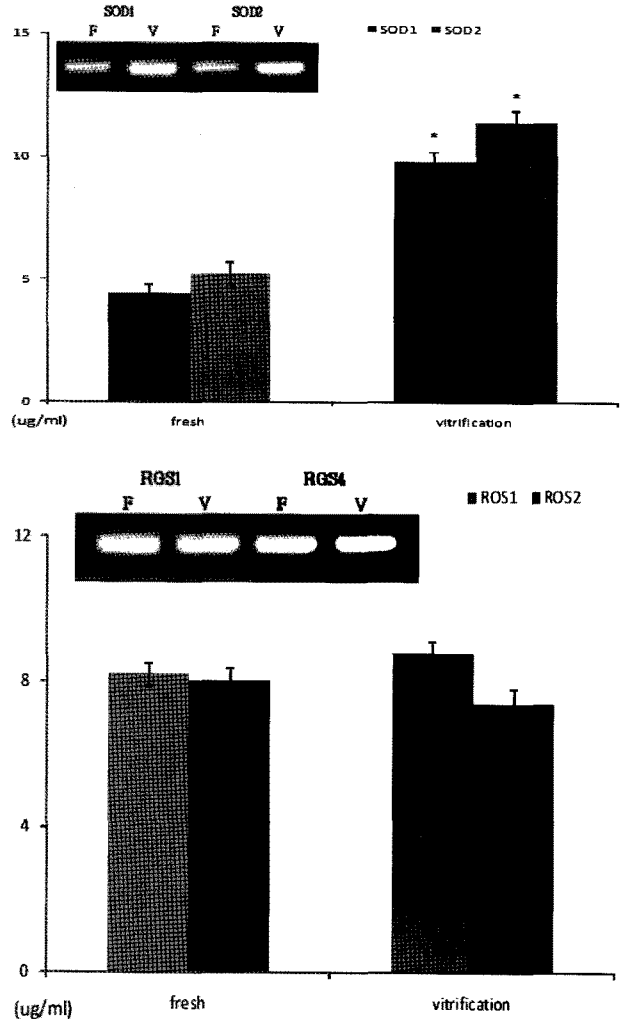

Fig. 3. SOD (up) and RGS (down) expression in fresh (F) and vitrified (V) immature oocytes. Individual expression profiles of genes in the *in vitro* derived oocytes. * Values within column with different superscript differ ($p<0.05$).

vage are reported to be between 8% and 37% in culture (Hewitt and England, 1997; Songsasen *et al.*, 2002; Rodrigues *et al.*, 2004). The low rates of canine embryonic development are a testament to the inefficient development of oocytes matured *in vitro*. The low rates of canine embryonic development are a testament to the inefficient development of oocytes matured *in vitro*.

The *in vitro* maturation rate of vitrified-thawed oocytes was 30.8±3.4%. The *in vitro* maturation rate of vitrified canine oocytes was lower than that of the control (52.0±2.5%, $p<0.05$). The *in vitro* maturation rate of vitrified-thawed oocytes was significantly ($p<0.05$) lower than those of fresh oocytes (Table 2). The *in vitro* maturation rates of the vitrified-thawed oocytes was 17.5 ±2.5%. These results were lower than those of the control group (43.6±3.2%). The *in vitro* developmental rates of the vitrified-thawed oocytes was 8.8±3.4% (Table 3). These results were lower than those of the control group (20.0±3.0%). SOD1 gene expression of 1~2 mm of follicle size were higher than those of above 6 mm follicle size. SOD2 gene expression of 1~2 mm of follicle size were significantly higher than those of above 6 mm follicle size ($p<0.01$). The expression pattern of SOD1, 2 was constantly expressed in both groups but strongly expressed in follicles (1~2 mm) group when compared to the above 6 mm follicles. Covarrubias *et al.* (2008) was reported the ROS are not always detrimental to the cells as they can carry out functional roles at certain concentrations, notably during embryonic development. SOD1 and SOD2 proteins and activity were elevated in follicular fluid from small follicles, while SOD1 protein appeared most abundant in oocytes and cumulus cells from large follicles relative to small follicles (Covarrubias *et al.*, 2008). As to the presence of SOD in the follicular fluid, it may provide protection for the maturing pig oocytes against oxidative damage, in turn improving developmental competence (Tatemoto *et al.* 2004), while supplementation with exogenous SOD failed to influence bovine IVM (Ali *et al.* 2003).

SOD gene expression between groups the fresh and vitrified oocytes groups were significant differences in rates. However, RGS gene expression between groups the fresh and vitrified oocytes groups were no significant differences in rates. The expression pattern of SOD and RGS did differ between fresh (control) and vitrified oocytes. For SOD gene was constantly expressed in both groups but strongly expressed in vitrified-thawed group when compared to the control. RGS gene was no differences in rates vitrified-thawed group when compared to the control (Fig. 2). Apoptosis is an underlying process in oocyte degeneration and embryo fragmentation. Contrarily, Dhali *et al.* (2000) showed that Bcl2 and BAX display decreased expression in vitrified mouse embryos compared to control. The expression and function of RGS4 have been studied in cardiomyocyte (Mittmann, 2002; Lee, 2005 and Hao *et al.*, 2006), nerve tissue (Krumins *et al.*, 2004) and cancers (Nikolova, 2008; Hurst *et al.*, 2009: Xie, 2009). The expression and function of RGS4 have been studied in nerve tissue (Krumins *et al.*, 2004). Gagon *et al.* (2002) have cloned a RGS18 transcript using degenerative oligonucleotides, that are predominantly expressed in platelets (Gagnon *et al.*, 2002). Although little is known about the exact mechanisms regulating the translation processes, one can assume that the differential expression of RGS transcription in rat platelets may result in the biosynthesis of corresponding RGS proteins. The present study demonstrated that it is possible to cryopreserve immature canine oocyte by vitrification using DES vitrification. Our results show that vitrified-thawed immature canine oocytes can resume meiosis and develop to the

MII stage. Further studies are required to investigate the fertility and developmental ability of MII oocytes following fertilization.

REFERENCES

1. Ali AA, Bilodeau JF, Sirard MA (2003): Antioxidant requirements for bovine oocytes varies during *in vitro* maturation, fertilization and development. Theriogenology 59:939-949.
2. Bedford SJ, Kurokawa M, Hinrichs K, Fissore RA (2003): Intracellular calcium oscillations and activation in horse oocytes injected with stallion sperm extracts or spermatozoa. Reproduction 126:489-499.
3. Berman DM, Gilman AG (1998): Mammalian RGS proteins: Barbarians at the gate. J Biol Chem 273 (3):1269-1272.
4. Clark P, Rahy GM, Karow Jr AM (1984): Factor influencing renal cryopreservation. II. Toxic effects of three cryopreservation in combination with three vehicle solutions in non-frozen rabbit cortical slices. Cryobiology 21:260-273.
5. Covarrubias L, Hernandez-Garcia D, Schnabel D, Salas-Vidal E, Castro-Obregon S (2008): Function of reactive oxygen species during animal development: passive or active? Developmental Biology 320:1-11.
6. Dhali A, Manik RS, Singla SK, Palta P (2000): Vitrification of buffalo (*Bubalus bubalis*) oocytes. Theriogenology 53:1295-1303.
7. Fahy GM, MacFarlane DR, Angell CA, Meryman HT (1984): Vitrification as an approach to cryopreservation. Cryobiology 21:407-426.
8. Gagnon AW, Murray DL, Leadley RJ (2002): Cloning and characterization of a novel regulator of G protein signalling in human platelets. Cell Signal, 14 (7):595-606.
9. Garcia A, Prabhakar S, Hughan S, Anderson TW, Brock CJ, Pearce AC, Dwek RA, Watson SP, Hebestreit HF, Zitzmann N (2004): Differential proteome analysis of TRAP activated platelets: Involvement of DOK-2 and phosphorylation of RGS proteins. Blood 103(6):2088-2095.
10. Hao J, Michalek C, Zhang W, Zhu M, Xu X, Mende U (2006): Regulation of cardiomyocyte signaling by RGS proteins: differential selectivity towards G proteins and susceptibility to regulation. J Mol Cell Cardiol 41:51-61.
11. Hewitt DA, England GCW. 1997. Effect of preovulatory endocrine events upon maturation of oocytes of domestic bitches. J Reprod Fertil (Supp), 151:83-91.
12. Hurst JH, Mendpara N, Hooks SB (2009): Regulator of G-protein signalling expression and function in ovarian cancer cell lines. Cell Mol Biol Lett 14:153-174.
13. Krumins AM (2004): Differentially regulated expression of endogenous RGS4 and RGS7. J Biol Chem 279:2593-2599.
14. Krumins AM, Barker SA, Huang C, Sunahara RK, Yu K, Wilkie TM, Gold SJ, Mumby SM (2004): Differentially regulated expression of endogenous RGS-4 and RGS7. J Biol Chem 279:2593-2599.
15. Lee MJ (2005): RGS4 and RGS5 are *in vivo* substrates of the N-end rule pathway. Proc Natl Acad Sci 102:15030-15035.
16. Martin CB, Mahon GM, Klinger MB, Kay RJ, Symons M, Der CJ, Whitehead IP (2001): The thrombin receptor, PAR-1, causes transformation by activation of Rho-mediated signaling pathways. Oncogene 20(16):1953-1963.
17. Mittmann C (2002): Expression of ten RGS proteins in human myocardium: functional characterization of an upregulation of RGS4 in heart failure. Cardiovasc Res 55:778-786.
18. Nikolova DN (2008): Genome-wide gene expression profiles of thyroid carcinoma: identification of molecular targets for treatment of thyroid carcinoma. Oncol Rep 20:105-121.
19. Otoi T, Shimizu R, Naoi H, Wongsrikeao P, Agung B, Taniguchi M (2005): Meiotic competence of canine oocytes embedded in collagen gel. Reprod Domest Anim 41:17-21.
20. Rodrigues BA, Carboneiro dos Santos L, Rodrigues JL (2004): Embryonic development of *in vitro* matured and *in vitro* fertilized dog oocytes. Mol Reprod Dev 67:215-223.
21. Ross EM, Wilkie TM (2000): GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem 69:795-827.
22. Songsasen N, Yu I, Leibo SP (2002): Nuclear maturation of canine oocytes cultured in protein-free media. Mol Reprod Dev 62:407-415.
23. Songsasen N, Wildt DE (2005): Size of the donor follicle, but not stage of reproductive cycle or seasonality, influences meiotic competency of selected domestic dog oocytes. Mol Reprod Dev 72:113-119.
24. Srinivasa SP, Bernstein LS, Blumer KJ, Linder ME (1998): Plasma membrane localization is required for RGS4 function in *Saccharomyces cerevisiae.* Proc Natl Acad Sci 95(10):5584-589.
25. Sutton RL (1992): Critical cooling rates for aqueous cryoprotectants in the presence of sugars and polysaccharides. Cryobiology 29:585-598.
26. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T (2004): Protection of porcine oocytes against cell damage caused by oxidative stress during *in vitro* maturation: role of superoxide dismutase activity in porcine follicular fluid. Biology of Reproduction 71:1150-1157.
27. Turathum B, Saikhun K, Sangsuwan P, Kitiyanant

Y (2010): Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrinol 22:70-83.
28. Utsumi K, Hochi S, Iritani A (1982): Cryopretective effect of polyols on rat embryos during two-step freezing. Cryobiology 29:332-341.
29. Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ (1996): RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature 383(6596):172-175.
30. Xie Y (2009): Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. Cancer Res 69:5743-5751.
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