氏名（本籍・生年月日）	HWANG In-Sul（大韓民国・1980 年 01 月 05 日）		
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論文審査委員	主査	教　授	保地　眞一
		教　授	下坂　誠
		准教授	片岡　正和
		准教授	堀江　智明
			菊地　和弘（(独)農業生物資源研究所）

論　文　内　容　の　要　旨

Successful pregnancies or birth of offspring derived from frozen-thawed oocytes have been reported in several mammalian species a few decades ago, with relatively low developmental rates. Application of vitrification, instead of the conventional two-step freezing, improved the efficacy of oocyte cryopreservation, especially in mice and humans. Various cryodevices have been developed to accelerate the cooling rate. However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement. During the vitrification procedures, oocytes can be damaged because of their large cell size and low permeability of water and CPA. Moreover, depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome misalignment. Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae. The objective of the present study was to investigate the effect of vitrification and warming on cryoinjuries of bovine MII oocytes, and to improve their revivability in terms of production of transferable high quality blastocysts by IVF and IVC.

A centrosome is composed of a pair of centrioles surrounded by the pericentriolar materials, such as γ-tubulin, centrin and pericentrin, and acts as the MTOC. In cattle, a sperm brings a centrosome into an oocyte during fertilization and a single sperm aster is formed by polymerization of α- and β-tubulin. The microtubule network plays a key role in the migration of male and female pronuclei to the center of a zygote and the subsequent fusion and mitotic cleavage. Additionally, timing of first cleavage in IVF derived bovine oocytes is important for yield and quality of blastocysts, as oocytes cleaving earlier are more likely to become blastocysts, and the resulting blastocysts have higher cryosurvival potential and higher pregnancy rates than those cleaving later. Thus, developmental kinetics can be used as a proxy of embryo quality. Therefore, profiles of cleavage and blastocyst development were first examined for vitrified bovine oocytes, and then function of MTOC/aster(s) in the vitrified oocytes after IVF was analyzed (CHAPTER II). The oocytes cleaved early can be developed to blastocysts at a higher rate than the oocytes cleaved later. Immunostaining for α-tubulin indicated that proportions of zygotes exhibiting aster formation were comparable between vitrified and fresh control groups. However interestingly, relative ratio of zygotes with a single aster vs multiple asters was significantly different between the two groups. Incidence of multiple aster formation in zygotes derived from vitrified oocytes was more than double that in zygotes derived from fresh control oocytes. Thus, we have proposed a new hypothesis for cryodamage of bovine oocytes that multiple aster formation frequently observed in vitrified- warmed

and fertilized oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly from the sperm-aster.

Increased apoptosis of vitrified-warmed oocytes resulted in reduction of developmental competence. The ROCK was discovered as a downstream target of the small GTP-binding protein Rho, which can regulate cellular growth, adhesion, migration, metabolism, and apoptosis through controlling the actin-cytoskeletal assembly and cell contraction. From stem cell researchers, it has been reported that inhibition of the ROCK activity was involved in reduction of apoptosis in embryonic stem cell-derived neural cells, and that inhibition of the ROCK activity was effective to improve the plating efficiency of dissociated human pluripotent stem cells after cryopreservation. Hochi et al. (2010) found that supplementation of ROCK inhibitor (Y-27632) to post-thaw culture medium for 48 h also significantly improved the revivability of in vitro-produced bovine blastocysts after vitrification and warming. Therefore, it was investigated whether short-term treatment of vitrified-warmed bovine oocytes with the ROCK inhibitor can improve the survival rate and the subsequent developmental competence after IVF (CHAPTER III). Treatment of the post-warm oocytes with 10 μM Y-27632 for 2 h resulted in the significantly higher oocyte survival rate prior to the IVF, cleavage rate and blastocyst formation rate. Quality analysis of the resultant blastocysts in terms of total cell number and apoptotic cell ratio also showed the positive effect of the Y-27632 treatment. Time-dependent change in mitochondrial activity of the vitrified-warmed oocytes was not influenced by ROCK inhibition during the period of recovery culture. However, the ability of ooplasm to support single-aster formation was improved by the ROCK inhibition. Thus, inhibition of ROCK activity in vitrified-warmed bovine oocytes during a short-term recovery culture can lead to the higher developmental competence, probably due to decreased apoptosis and normalized function of MTOC.

In conclusion, vitrification of bovine matured oocytes increased the formation of multiple sperm asters after IVF which less contributed to the migration and development of pronuclei. This is a new hypothesis for injuries induced in the vitrified-warmed bovine oocytes. Interestingly, inhibition of ROCK activity during a short-term recovery culture prior to IVF had a beneficial effect on the developmental competence (both blastocyst yield and quality) of the vitrified-warmed bovine oocytes. These results would push forward the cryo-banking of unfertilized oocytes in the bovine species.