Vitrification procedure decreases inositol 1,4,5-triphosphate receptor expression, resulting in low fertility of pig oocytes

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Although cryopreservation of mammalian oocytes is an important technology, it is well known that unfertilized oocytes, especially in pigs, are highly sensitive to low temperature and that cryopreserved oocytes show low fertility and developmental ability (Wu *et al.* 2006). To avoid these detrimental effects caused by exposure to low temperature, many attempts have been done. It is recognized that one of the important issues contributing to successful vitrification is to maximize the rate of cooling and warming (Kuwayama *et al.* 2007). In our previous study, we demonstrated that Cryotop yields high post-warming survival and developmental ability of vitrified oocytes in the mouse (Kohaya *et al.* 2011) and rat (Fujiwara *et al.* 2010).

As for vitrification of metaphase-II (MII) oocytes, it is thought that kinetics of intracellular calcium is also an essential factor for the success. During fertilization, rises of intracellular calcium released from the endoplasmic reticulum via inositol 1,4,5-triphosphate receptor, (IP₃R1) occur in oocytes (Ito et al. 2010; Ito & Kashiwazaki 2012). The rises of intracellular calcium induce exocytosis of cortical granules, resulting in the occurrence of zona hardening known as the zona reaction (Ducibella et al. 1988). In the mouse, Larman et al. (2006) demonstrated that exposure to vitrification solution containing cryoprotective agents causes the rise of intracellular calcium in MII oocytes, inducing zona hardening as well as normal fertilization. Indeed, our previous data showed that vitrification using calcium-free media significantly improved penetration and fertilization rates of vitrified oocytes in the mouse (Kohaya et al. 2011) and rat (Fujiwara et al. 2010). However, in pigs, the effect of calcium during vitrification in not well examined. In the present study, we focused on the kinetics of IP₃R1 during vitrification in pig oocytes and tried to clarify the reason why vitrified pig oocytes show low fertility and developmental ability.

Cumulus oocyte complexes (COCs) were cultured for 44 h and then used for vitrification. Vitrification-warming was carried out by the Cryotop method as previously described (Fujiwara et al. 2010). Vitrified-warmed COCs were used for *in vitro* fertilization (IVF). For immunostaining, the germinal vesicle (GV) stage and MII stage oocytes were collected at 0 h and 44 h, respectively. For western blotting, GV, MII, exposed MII and vitrified-warmed MII oocytes were collected. *In vitro* matured COCs were vitrified with Cryotop and then evaluated for fertility through IVF. Pronuclear formation rates of vitrified-warmed oocytes were significantly (p < 0.05) lower regardless of calcium supplementation (9.0 \pm 1.3% (Ca+) and 8.0 \pm 2.6% (Ca-)) compared to that of fresh ones (62.4 \pm 13.0%). Most of the fertilized fresh oocytes reached the 2-cell stage (53.3 \pm 10.1%). Some of the 2-cell embryos also developed to blastocysts (22.4 \pm 1.8%). However, none of the fertilized oocytes in the vitrified groups developed to the 2-cell

stage. The penetrated sperm was confirmed in most of the oocytes as failing to form pronuclei, suggesting the possibility that vitrified-warmed oocytes have a decreased activity to induce calcium oscillations. To clarify the reason why vitrified-warmed oocytes had low fertility after IVF, localization of IP₃R1 in vitrified-warmed oocytes was examined. In fresh MII oocytes, IP₃R1 is widely localized in oocyte cytoplasm and at the plasma membrane. Exposure to equilibration and vitrification media did not affect the IP₃R1 localization. However, in vitrified oocytes, no localization of IP₃R1 at the plasma membrane could be visualized. These results suggest that the vitrification procedure may affect the expression of IP₃R1 in pig oocyte. Therefore next, we tried to clarify whether the vitrification procedure affected IP₃R1 expression in pig oocytes at the protein levels using western blotting. There were no significant differences in expression levels of IP₃R1 and β -actin between GV and MII oocytes. In addition, exposure of oocytes to equilibration and vitrified-warmed oocytes, a significant decrease of IP₃R1 expression was observed, while expression of β -actin was only slightly decreased.

Taken together, our present results demonstrate a possibility that the low fertility and developmental ability of vitrified MII porcine oocytes are due to a decreased expression of IP₃R1 and its abnormal localization.

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