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ARTICLE

Effects of vitrification solutions and equilibration times on the morphology of cynomolgus ovarian tissues

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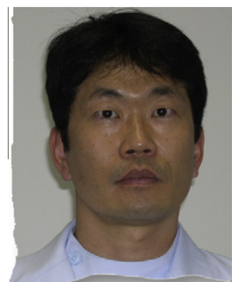
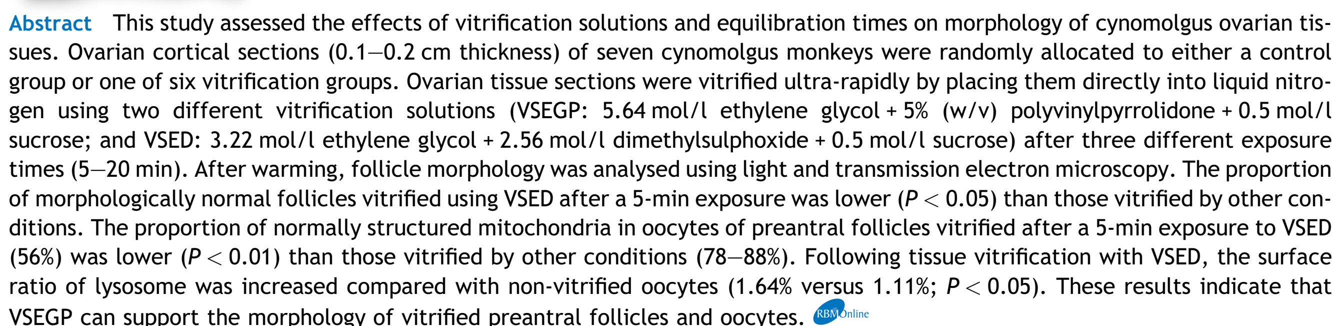


Dr Hashimoto obtained his PhD in Reproductive Physiology at Kyoto University in 2001. He started his research career in 1989 by production of transgenic mice, rat, rabbits and cattle. He also developed assisted reproduction technology in cattle (superovulation, transvaginal oocyte retrieval, in-vitro maturation, IVF, intracytoplasmic sperm injection, embryo culture and vitrification) at Snow Brand Milk Products. He moved to IVF Namba Clinic in 2004 and started to work on human assisted reproduction. Currently, he is the research director of IVF Namba Clinic. He was recently secretary-general of the Japan Society of Fertilization and Implantation. He received the Japanese Society of Animal Reproduction innovative technology award in 2008 and the Japanese Society of Mammalian Ova Research outstanding presentation award in 2009.



Abstract This study assessed the effects of vitrification solutions and equilibration times on morphology of cynomolgus ovarian tissues. Ovarian cortical sections (0.1–0.2 cm thickness) of seven cynomolgus monkeys were randomly allocated to either a control group or one of six vitrification groups. Ovarian tissue sections were vitrified ultra-rapidly by placing them directly into liquid nitrogen using two different vitrification solutions (VSEGP: 5.64 mol/l ethylene glycol + 5% (w/v) polyvinylpyrrolidone + 0.5 mol/l sucrose; and VSED: 3.22 mol/l ethylene glycol + 2.56 mol/l dimethylsulphoxide + 0.5 mol/l sucrose) after three different exposure times (5–20 min). After warming, follicle morphology was analysed using light and transmission electron microscopy. The proportion of morphologically normal follicles vitrified using VSED after a 5-min exposure was lower ($P < 0.05$) than those vitrified by other conditions. The proportion of normally structured mitochondria in oocytes of preantral follicles vitrified after a 5-min exposure to VSED (56%) was lower ($P < 0.01$) than those vitrified by other conditions (78–88%). Following tissue vitrification with VSED, the surface ratio of lysosome was increased compared with non-vitrified oocytes (1.64% versus 1.11%; $P < 0.05$). These results indicate that VSEGP can support the morphology of vitrified preantral follicles and oocytes. RBMOnline

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KEYWORDS: autolysosome, lysosome, mitochondria, ultra-rapid vitrification

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Introduction

Survival rates for cancer patients have been increasing due to the constant progress in the diagnosis and treatment of the disease (Lutchman Sigh et al., 2005). However, women requiring chemotherapy and/or radiotherapy for cancer, leukaemia or other benign pathologies are likely to experience premature ovarian failure and loss of fertility as a consequence of these potentially gonadotoxic treatments. Several options are currently available to preserve fertility in these patients, giving them the choice to become mothers when they have overcome their disease: cryopreservation of embryos, oocytes or ovarian tissues (Jeruss and Woodruff, 2009). Selection of the most suitable strategy for preserving fertility depends on various parameters: the type and timing of chemotherapy, the type of cancer, the patient's age and her partner's status.

Several studies have shown that ovarian tissue can be successfully frozen and grafted (Callejo et al., 2001; Demeestere et al., 2006; Donnez et al., 2004, 2006; Fabbri, 2006; Gook et al., 2001, 2003, 2005; Kim et al., 2003, 2004, 2009; Meirow et al., 2005, 2007; Nottola et al., 2008; Oktay and Karlikaya, 2000; Oktay et al., 2004; Schmidt et al., 2005; von Wolff et al., 2009). Although human births have recently been reported following cryopreserved ovarian autograft (Andersen et al., 2008; Demeestere et al., 2007; Donnez et al., 2004; Meirow et al., 2005), the technique cannot yet be considered a part of current medical practice. The size of ovarian tissue is problematic and results in intracellular ice formation due to the difficulty of adequate diffusion of cryoprotective agents into large tissue masses. However, successful ovarian cryopreservation is a particularly important goal because ongoing progress in cancer treatment is resulting in more women surviving cancers that are diagnosed early in their reproductive life (Linet et al., 1999).

Two main methods are used to cryopreserve biological tissue: slow freezing and vitrification by rapid cooling. The slow cooling method uses an optimal cooling rate specific to a cell but produces extracellular ice crystals that are harmful to the surrounding tissue. Isachenko et al. (2007) reported that slow freezing is more successful than rapid freezing for the cryopreservation of human ovarian tissue. However, vitrification conditions can range widely. In addition, vitrification traps all the aqueous solutions in an amorphous, so-called vitreous, solid phase, preventing any ice crystal formation in the tissue (Pegg, 2001). Thus, slow freezing may preserve a few cells without damage, but vitrification seems to be preferable for the cryopreservation of more complex and heterogeneous systems such as ovarian cortex tissues. Recently, ultra-rapid cooling methods, which have been developed using mice, have been shown to support the viability of vitrified ovarian tissues (Chen et al., 2006; Wang et al., 2008). In addition, Keros et al. (2009) found that the viability of stroma cells was much better using vitrification compared with controlled-rate freezing.

However, vitrification conditions have not yet been well determined. Vitrification requires rapid cooling in combination with a high yet subtoxic concentration of cryoprotectants to produce an efficient glass-forming mixture with water. This study investigated the effects of vitrification solutions and equilibration times on the morphology of vitrified preantral follicles in primates.

Materials and methods

The study protocol was approved by the ethics committee at the IVF Namba Clinic and was performed in accordance with the guidelines set out for use at the experimental facilities at Shin Nippon Biomedical Laboratories (Japan), from which ovaries were obtained from seven healthy parous cynomolgus monkeys (85–137 months old, which corresponds to 39–48 years. Tissues were placed in saline in a thermos bottle at 35°C and transported in an insulated bottle within 5 h from Kagoshima to the laboratory, where the ovaries were trimmed from adhering tissue and then washed twice in HEPES-buffered 199 (H199, 12350–039; Invitrogen, Tokyo, Japan) supplemented with 20% (v/v) serum substitute supplement (SSS, 99193; Irvine Scientific, St. Ana, CA, USA). Cortical tissue was then removed from each pair of ovaries and cut into small sections (within $1 \times 1 \times 0.1$–0.2 cm; **Figure 1**) to maximize surface ratio per volume. Next, the ovarian sections were placed in H199 supplemented with 20% SSS at room temperature (24–26°C). Ovarian sections (sized within $10 \times 10 \times 1$ mm) were randomly distributed over the experiments.

Vitrification

Two types of vitrification solutions were compared. The first vitrification solution of ethylene glycol (EG) and dimethylsulphoxide (DMSO) (VSED; Ishimori et al., 1992) contained H199 supplemented with 20% SSS, 3.22 mol/l EG (054–0983; Wako Chemical, Osaka, Japan), 2.56 mol/l DMSO (D2650; Sigma–Aldrich, St Louis, MO, USA) and 0.5 mol/l sucrose (192–00012; Wako Chemical). The second vitrification solution of EG and polyvinylpyrrolidone (PVP) (VSEGP; Gandolfi et al., 2006) contained H199 supplemented with 20% SSS, 5.64 mol/l EG, 5% (w/v) (PVP 360; Sigma) and 0.5 mol/l sucrose. Three stepwise equilibrations were used for the vitrification of ovarian tissues. For VSED, ovarian cortex sections were exposed to H199 supplemented with 20% SSS, 0.805 mol/l EG and 0.64 mol/l DMSO for 10 min, transferred into H199 supplemented with 20% SSS, 1.61 mol/l EG and 1.28 mol/l DMSO for 10 min and equilibrated in VSED for 5, 10 or 20 min at room temperature (24–26°C). Ovarian sections equilibrated with VSED were then loaded on the Cryosupport (**Figure 1**) using tweezers and immersed vertically into liquid nitrogen (−196°C) directly with minimum volume of VSED. Tissues were in direct contact with liquid nitrogen during the vitrification procedure. The Cryosupport consisted of four fine stainless needles and a cryogenic vial (32815; BD Falcon, NJ, USA). For VSEGP, ovarian cortex sections were exposed to H199 supplemented with 20% SSS and 1.61 mol/l EG for 10 min, transferred into H199 supplemented with 20% SSS and 3.22 mol/l EG for 10 min and equilibrated in VSEGP for 5, 10, or 20 min at room temperature. Ovarian sections equilibrated with VSEGP were loaded on the Cryosupport and immersed vertically into liquid nitrogen in a similar manner to that used for VSED samples. These vitrified ovarian sections were stored in liquid nitrogen for at least 2 months

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