IMPORTANCE OF PRESERVATION OF GAMETES AND GONADAL TISSUE FOR PORCINE GENETIC RESOURCES

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ABSTRACT

The conservation or preservation of mammalian genetic resources, especially farm animals, has been conducted under in situ *conditions by maintaining living individuals as "livestock." However, systems for laboratory* in vitro *embryo production using gametes such as spermatozoa and oocytes are now available and* in vitro *culture for embryos viable to offspring after transfer to recipients, as an* ex situ *preservation method for mammalian genetic resources. In pigs, freezing of sperm is the most reliable and well-established method for this purpose. On the other hand, cryopreservation of female gametes (oocytes) and gonadal (testicular and ovarian) tissues usually by vitrification has been conducted; however, resulted in very low efficacies. Recently, our laboratory conducted some research themes related to these issues. We have been focusing on advances using porcine* in vitro *embryo production (IVP) systems and xenografting into host immunodeficient mice for piglet generation. In this symposium, we introduce recent progress on the vitrification of porcine immature oocytes and gonadal tissues followed by their IVP and xenografting to produce gametes.*

Keywords: Pig, Cryopreservation, Vitrification, Oocyte, Gonadal tissue, Gene bank

INTRODUCTION

The main type of genetic material for routine cryopreservation by gene banking is spermatozoa, that is, so called semen cryobanking. Banking of other materials such as oocytes or early embryos has been considered challenging. However, the utilization of female gametes and gonadal tissues has been considered very important. Some attempts at vitrification of tissues have been made, and for this purpose, *in vitro* embryo production (IVP) and its related technologies are essential.

The basic IVP procedure for piglet production has been of fundamental importance in studies of embryo freezing/vitrification at the pronucleus (Somfai *et al*. 2009), the 4- to 8-cell (Nagashima *et al*. 2007), the morula (Maehara *et al*. 2012), and the blastocyst (Mito *et al*. 2015) stages. And intracytoplasmic sperm injection (ICSI)-related technologies (Nakai *et al*. 2003, 2009, 2010, Kaneko *et al*. 2013) are also fundamental for the utilization sperm without self-penetration ability into oocytes.

CRYOPRESERVATION OF UNFERTILIZED OOCYTES

Freezing of spermatozoa is one of the basic approaches for preservation of genetic resources. Oocyte cryopreservation combined is a basic strategy for gene banking of female germplasm; for pigs, however, this technology is very difficult and still considered a challenge.

We have tested the possibility of vitrification for immature (at the germinal vesicle stage; cumulus oocyte complexes) and mature (at the metaphase-II stage) oocytes before fertilization (Somfai *et al.* 2012). The survival rate for mature oocytes was relatively high; however, a high proportion (about 70%) of vitrified immature oocytes were able to complete meiotic maturation and undergo normal fertilization and embryo development. Using cryopreserved immature oocytes, we had focused on piglet production after *in vitro* maturation/fertilization (IVM/IVF) and embryo transfer, and finally obtained. piglets (Somafi *et al.* 2014).

This application has been confirmed using immature oocytes from commercial Western pigs, which seems to be also efficient reproduction system in native species especially, in Asian countries. We have conducted under the project by Science and Technology Research Partnership for Sustainable Development (SATREPS) for Vietnamese native pigs (https://www.jst.go.jp/global/english/kadai/h2604_vietnam.html) and found equal efficacy in cryopreservation efficacies (Somfai *et al*. unpublished data).

CRYOPRESERVATION OF GONADAL TISSUES

Testicular tissue

One approach for inducing spermatogenesis in isolated testicular tissues is ectopic (into another site in the body) grafting into immunodeficient host animals (xenografting). Ectopic xenografting has been reported for hamsters (Schlatt *et al*. 2002), goats (Honaramooz *et al*. 2002), rhesus macaques (Honaramooz *et al*. 2004), sheep (Zeng *et al*. 2006), cats (Snedaker *et al*. 2004), and pigs (Nakai *et al*. 2009, Nakai *et al*. 2010, Honaramooz *et al*. 2002, Zeng *et al*. 2006, 2007, Honaramooz *et al*. 2008, Kaneko *et al*. 2008). It is preferable to graft testicular tissue under the skin of the back of commercially available severe combined immunodeficiency mice or nude mice.

A series of studies has been conducted in our laboratory (Nakai *et al*. 2009, Nakai *et al*. 2010, Kaneko *et al*. 2008, Kikuchi *et al*. 2011) to evaluate whether boar spermatogonia can develop into sperm in testicular tissues grafted into immunodeficient nude mice, and whether live piglets can be produced from this sperm by ICSI into oocytes. Elongated spermatids and spermatozoa were obtained after 4 months. Just after collection, some spermatozoa showed only faint motility for a short period; however, they could not penetrate into IVM oocytes, meaning that we unfortunately could not apply these sperm for IVP and have to select ICSI method to obtain fertilized oocytes. After culture, some of them developed into blastocysts; which were similar to the oocytes from prepubertal gilts after IVM/IVF (Kikuchi *et al*. 2002, Nakai *et al*. 2009]. When oocytes at the pronuclear stage after ICSI were transferred to oviducts of estrous-synchronized recipients, we were able to obtain both male and female piglets (Nakai *et al*. 2010), which showed normal reproductive abilities when developed to the adulthood (Kaneko *et al*. 2012).

For more advanced utilization of this technique, we have investigated the possibility of vitrification of testicular tissue fragments before xenografting. This method enables long term storage in liquid nitrogen of the tissue and the production of sperm whenever the need arises. After a certain period (120 days), sperm were first recovered from the grafts. The sperm recovery rate increased with time after grafting from 180 to 350 days. Sperm after the recovery from the tissues were applied for ICSI, and the oocytes were transferred to recipients. We could obtain live male and female piglets (Kaneko *et al*. 2013). Both the male and female pigs showed normal reproductive abilities (Kaneko *et al*. 2014).

Ovarian tissues

Primordial follicles act as stores for ovarian follicles and can be potential sources of oocytes. To utilize these resources, ovarian tissue grafting is a possible method for growing or maturing such primordial oocytes in the primordial follicles of large mammals. In humans and primates, grafting of ovarian tissues to another site in the body (autografting) has been used successfully to produce viable offspring (Lee *et al*. 2004, Donnez *et al*. 2004). Xenografting is more advantageous for the conservation and multiplication of domestic or endangered animals. Some studies have been conducted in cat (Gosden et al. 1994), sheep (Gosden *et al*. 1994), mice (Snow *et al*. 2002), humans, (Oktay *et al*. 1998, Weissman *et al*. 1999, Kim *et al*. 2002, Gook *et al*. 2003), dogs (Metcalfe *et al*. 2001), monkeys (Candy *et al*. 1995), cattle (Senbon *et al*. 2003), pigs (Kaneko *et al*. 2003), tammar wallabies (Mattiske *et al*. 2002), and common wombats (Cleary *et al*. 2003, 2004).

Our studies using pigs were the first to demonstrate clearly that such oocytes were competent in terms of fertilization ability (Kaneko *et al*. 2003) and had limited potential for development into embryos (Kaneko *et al*. 2006, Kikuchi *et al*. 2006, 2011); however, we did not have not yet obtain any piglet. From the viewpoint of genetic resource conservation, cryopreservation of ovarian tissues before xenografting may be important. We are trying to confirm this possibility and reported the fertilizing ability (Kikuchi *et al*. 2010), but its efficacy still remains quite low.

CONCLUSION AND PERSPECTIVE

Although pigs are important as a source of meat, they are also expected to have considerable potential as models or donors in human regenerative medicine. For example, the possibility of generating the human pancreas from human induced pluripotent stem cells using genetically modified pigs has been suggested. For these purposes, the conservation of porcine genetic resource is very important. We are now encouraged to establish the conservation technologies in pigs.

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