Theriogenology 80 (2013) 887–892

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Improved developmental ability of porcine oocytes grown in nude mice after fusion with cytoplasmic fragments prepared by centrifugation: A model for utilization of primordial oocytes

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ARTICLE INFO

Article history:
Received 18 April 2013
Received in revised form 7 July 2013
Accepted 23 July 2013

Keywords:
Primordial oocyte
Xenografting
Cytoplasm fusion
In vitro embryo production
Pig

ABSTRACT

Primordial oocytes are a potential resource for medical and zoological application, but those of large animals have not yet been reported to show efficient embryonic development. In the present study, we established a pig model for production of blastocysts from primordial oocytes that had been grafted into nude mice and matured *in vitro*, in combination with fusion of cytoplasmic fragments. Neonatal porcine ovaries in which most follicles are at the primordial stage were minced and grafted into nude mice (Crlj:CD1-*Foxn1*^{nu}). About 60 days after detection of vaginal opening, the mice were given 62.5 U/mL porcine FSH for 2 weeks by infusion to enhance follicular development. Developmentally competent oocytes collected from porcine ovaries (conventional oocytes) were matured *in vitro* and subjected to serial centrifugation to prepare cytoplasmic fragments without a metaphase plate (cytoplasts). Three cytoplasts were fused by electrostimulation to an oocyte retrieved from a host mouse (xenogeneic oocyte) and matured *in vitro*. Then these fused oocytes were fertilized and subsequently cultured *in vitro*. No blastocysts were generated from xenogeneic oocytes without fusion of cytoplasm. When xenogeneic oocytes had been fused with three cytoplasts, the blastocyst rate increased significantly to 14.3%, comparable to that for untreated conventional oocytes (20.0%). The numbers of cells in blastocysts for these fused oocytes (37.2 cells/blastocyst) were not significantly different from those for conventional oocytes (25.4 cells/blastocyst). Our findings show that it is possible to use primordial oocytes of large mammals in combination with xenografting of ovarian tissue and also ooplasmic fusion.

1. Introduction

Oocytes in primordial follicles (primordial oocytes) are a potentially useable resource for preservation of fertility, especially in the medical field for young cancer survivors [1–3] or in agriculture and zoology for genetic preservation [4,5]. In 1994, Gosden et al. [6] first demonstrated that sheep ovarian grafts not only survive for many months in mice with severe combined immunodeficiency, but also have follicles that develop to the antral stage. That pioneering study demonstrated the potential applicability of this model to a wide range of animals, and that follicles developing in the xenografts may contain fertile oocytes. In the agricultural and zoological fields, ovarian tissues prepared from many species have been grafted into immunodeficient mice [4,5], and in several species

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0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.07.014

primordial oocytes have been reported to grow and acquire maturation (pigs [7–9], cattle [10], cats [11]) and fertilization (pigs [7–9], cattle [10]) ability *in vitro*. On the other hand, xenografting of ovarian tissue can also yield basic data about the potential activity of immature oocytes, especially human primordial oocytes. Grafting of ovarian fragments prepared from women [12–15] or fetuses [16] into recipient mice has been reported to result in the development of antral follicles [16] and resumption of oocyte meiosis [12–15]. To date, however, no fetal development or birth after the transfer has yet been obtained after ovarian xenografting of species phylogenetically distant from mice.

We have used neonatal pigs as a model for evaluating the developmental ability of primordial oocytes in large mammals [7–9]. In ovaries of pigs at 20 days' postpartum, primordial follicles account for 96% of the total number of follicles and the rest are mostly primary follicles [7], a situation that is strikingly different from that in other large mammals such as humans, cattle, and sheep, which develop antral follicles in the late fetal period [17]. Therefore, the neonatal pig appears to a good model for investigating the growth and functional maturation of primordial oocytes after xenografting because the large population of primordial oocytes can be manipulated without any effects from more advanced follicles.

One strategy for improving the developmental competence of oocytes within xenografts is to facilitate oocyte development by accelerating follicular growth with exogenous hormones. Beneficial effects of gonadotropin treatment on the survival of follicles have been noted in human xenografts [12–15,18,19]. Our previous xenografting studies using neonatal pigs [7,8] revealed that gonadotropin treatment markedly increased the numbers of oocytes with IVM and IVF ability, although the blastocyst formation rate after IVF was less than 1% [8]. Furthermore, these IVF oocytes failed to produce blastocysts when they were transferred to the oviducts of estrous-synchronized recipient gilts [9]. Taken together, the existing data suggest that xenogeneic oocytes in large mammals are able to attain nuclear maturation, but seem to have difficulty in achieving full cytoplasmic maturation to support embryonic development. Another strategy for xenogeneic oocytes is to directly manipulate their cytoplasmic maturity by nuclear transfer. Transfer of a nucleus from a poor-quality oocyte at the germinal vesicle or metaphase II stage into an enucleated oocyte with full developmental competence has been reported to produce embryos in mouse [20–22], human [23], and cattle [24]. However, nuclear transfer requires equipment for micromanipulation, and the efficiency of the technique depends considerably on the skill of the operator.

Recently, we have established the "centri-fusion" method originally used for producing somatic nuclear transfer embryos in pigs [25]. This method makes it possible to obtain cytoplasmic fragments without a metaphase plate (cytoplasts) or with a metaphase plate (karyoplasts) from IVM oocytes simply by serial centrifugation: such cytoplasts have reportedly been fused to a somatic cell to produce an embryo [25] or cytoplasm and a karyoplast has been reported to produce a reconstructed oocyte [26]. Theoretically, fusion of cytoplasts prepared from developmentally competent oocytes is expected to improve the developmental ability of low-quality oocytes by increasing their cytoplasmic maturity. In fact, Linh et al. [27] demonstrated that IVM oocytes showing low developmental ability obtained under unsuitable culture conditions recovered their ability to produce an embryo after they had been fused with cytoplasts prepared from fully matured oocytes. We therefore prepared cytoplasts from oocytes that had been obtained from porcine ovaries and matured *in vitro* and examined whether fusion with these cytoplasts would improve the developmental competence of xenogeneic porcine oocytes in an *in vitro* embryo production system.

2. Materials and methods

2.1. Experimental design

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Japan. Porcine oocytes collected from host mice (xenogeneic oocytes) and pigs (conventional oocytes) were matured *in vitro*. Cytoplasts were prepared from conventional oocytes by the centri-fusion method described in the following section. Conventional oocytes unfused with cytoplasts were used to assess the developmental ability of oocytes that had grown *in situ* (normally in pigs) (CONV+0C group). Conventional oocytes were also each fused with three cytoplasts for confirmation of the fusion process (CONV+3C group). For experimental groups, xenogeneic oocytes were unfused with cytoplasts (XENO+0C group) or fused with three cytoplasts (XENO+3C). Oocytes in each group were subjected to IVF and then the subsequent blastocyst formation rates were compared among the groups.

2.2. Animals

Sixty female nude mice (Crlj:CD1-*Foxn1*^{nu}; Charles River Japan, Yokohama, Japan) age 5 to 6 weeks were used as recipients for the xenografts. They were kept in an environmentally controlled room (Koito, Yokohama Japan) maintained at a temperature at 24 °C and a humidity of 50% and illuminated daily from 5 AM to 7 PM. The mice fed at *ad libitum*.

2.3. Xenografting of porcine ovarian tissues

Ovaries were obtained after slaughter from 10 piglets aged 20 days (crossbreeds of Landrace × Large White × Duroc, born at the National Institute of Livestock and Grassland Science, Japan). Cortices of ovaries were dissected out and then minced into pieces measuring approximately 1.5 × 1.5 × 1.5 mm, as reported previously [7]. At this age, previous histological examinations had shown that primordial follicles accounted for 96% of the total number of follicles in the ovary and that the rest were almost all primary follicles [7]. The recipient mice were anesthetized with a combination of pentobarbital sodium (Somnopentyl; Kyoritsu Pharmaceuticals, Tokyo,