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Normal reproductive development of pigs produced using sperm retrieved from immature testicular tissue cryopreserved and grafted into nude mice

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ABSTRACT

Xenografting of immature testicular tissue combined with cryopreservation can preserve and use genetic information of prepubertal animals. For establishment of this new approach, it is essential to clarify whether offspring derived from sperm grown in host mice harboring cryopreserved xenografts show normal reproductive development. This study examined serum profiles of gonadal hormones during sexual maturation in pigs generated by intracytoplasmic sperm injection using sperm derived from cryopreserved xenografts (CryoXeno pigs; three males and three females). We also assessed the reproductive abilities of the male CryoXeno pigs by mating them with conventionally produced (conventional) pigs, and by examining the *in vitro* fertilizing ability of their sperm. For female CryoXeno pigs, reproductive ability was evaluated by artificial insemination with semen from a conventional boar. During the growth of male CryoXeno pigs, the serum concentrations of inhibin and testosterone showed similar changes ($P > 0.17$) to those in conventional pigs ($n = 4$). Histologic analyses of the testes revealed no differences ($P > 0.2$) in the growth and differentiation of seminiferous tubules between CryoXeno and conventional pigs. Three conventional sows delivered 13.0 ± 1.0 (mean $\pm$ standard error of the mean) live piglets after being mated with the three CryoXeno males. Sperm obtained from all CryoXeno pigs had the ability to penetrate oocytes, and these fertilized oocytes reached the blastocyst stage *in vitro*. During the growth of female CryoXeno pigs, the serum inhibin profile was similar ($P > 0.17$) to that observed in conventional pigs ($n = 5$). The first rise in serum progesterone concentration to more than 2 ng/mL was noted at 32.0 ± 2.3 weeks of age in the CryoXeno pigs and at 32.0 ± 3.3 weeks in the conventional pigs, suggesting that both pigs reached puberty at a similar age. After puberty, female CryoXeno pigs farrowed 8.3 ± 1.7 (mean $\pm$ standard error of the mean; $n = 3$) live piglets after artificial insemination with semen from a conventional boar. In conclusion, these findings demonstrate that both male and female CryoXeno pigs have normal reproductive abilities.

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1. Introduction

Honaramooz, et al. [1] first reported a novel method for completion of spermatogenesis by grafting fresh testicular

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tissue from neonatal donors into immunodeficient mice. Although so far limited to rabbits [2] and pigs [3], using sperm obtained from testicular grafts grown in host mice, intracytoplasmic sperm injection (ICSI) has been used successfully to produce live offspring with normal reproductive potential [4]. Thus, xenografting of fresh testicular tissue combined with ICSI makes it possible to obtain a new generation from young donors that have not yet reached sexual maturation and cannot be used for reproduction. However, when applying these techniques for conservation of genetically valuable animals such as rare breeds, endangered species, or those with genetic modifications that result in neonatal lethality, testicular tissues will often need to be stored until offspring production becomes necessary. Therefore, effective preservation of donor testis has been indispensable for practical application.

For this purpose, cryopreservation has been applied to neonatal or juvenile testicular tissues of several species, including human [5,6], primates [7–9], pig [1,10–12], and rabbit [2], and the ability of the cryopreserved tissue to complete spermatogenesis has been assessed by xenografting it into immunodeficient mice. Tissue stored in liquid nitrogen after slow freezing using a low concentration of cryoprotectant such as DMSO, glycerol or ethylene glycol maintains its capacity to produce spermatocytes (human [6], primate [8]), spermatids (pig [10,11]) or sperm (pig [1,12], rabbit [2]). Cryopreservation by vitrification, which involves ultra-high cooling rates in the presence of very high concentrations of cryoprotectants, has also been shown to allow testicular tissue to produce spermatocytes (human [6]) or sperm (pig [12]). Recently, we also cryopreserved testicular tissue from neonatal piglets after vitrification using a mixture of ethylene glycol, polyvinyl pyrrolidone, and trehalose as cryoprotectants, and then grafted the tissue into nude mice; this resulted in the first successful production of viable piglets by ICSI using sperm derived from cryopreserved immature testis [13]. To establish this new system consisting of cryopreservation, xenografting, and ICSI, there is a need to determine whether offspring derived from cryopreserved testicular xenografts can grow to sexual maturation. In the present study, therefore, we aimed to (1) compare hormonal profiles in relation to sexual maturation between pigs produced by the new system (CryoXeno pigs, [13]) and those produced with conventional artificial insemination (AI) (conventional pigs); (2) assess the reproductive ability of CryoXeno pigs by confirming the production of piglets after mating or AI with conventional pigs; and (3) for males, analyze their testicular histology and the *in vitro* fertilizing ability of the sperm after freezing and thawing.

2. Materials and methods

2.1. Pigs generated using sperm derived from immature testis after cryopreservation

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Japan. A previous study by our team had produced three female and four male piglets using sperm derived from testicular xenografts (donor; Landrace × Large White × Duroc crossbreed) after long-term cryopreservation for more than 140 days [13]. Six (three males and three females) of seven CryoXeno pigs were used in the present study. Their body weight at birth was 0.9 ± 0.1 kg (mean ± standard error of the mean [SEM]). These animals were subjected to blood sampling during sexual maturation, and then all of them were used for breeding after puberty. Finally, all males were slaughtered at a local abattoir for collection of testes and epididymal sperm.

2.2. Blood sampling

Blood samples were obtained from the auricular vein of the six CryoXeno pigs at 5, 10, 14, 19, 24, 28, 32, 37, 41, and 46 weeks after birth, to characterize the serum profiles of gonadal hormones such as inhibin, testosterone, and progesterone, which reflect sexual maturation. For comparison, blood samples were also obtained from four conventional males and five females (Landrace × Large White × Duroc crossbreed, born at the National Institute of Livestock and Grassland Science, Tsukuba, Japan) at the same weeks of age as the CryoXeno pigs.

2.3. Breeding

Three male CryoXeno pigs between 1.3 and 2.0 years of age were mated with three conventional gilts (Landrace × Large White crossbreed) purchased commercially (CIMCO Co. Ltd., Tokyo, Japan). The three female CryoXeno pigs were inseminated artificially with fresh Duroc semen purchased commercially (CIMCO) between 1.3 and 1.6 years of age. The number and body weights of the piglets delivered were recorded.

2.4. Collection of testes and epididymal sperm

Pairs of testes and epididymides were collected from the three male CryoXeno pigs slaughtered between 2.1 and 2.5 years of age. Three pieces, excised from different portions of the left testis of each pig, were fixed in Bouin solution and embedded in paraffin for histologic examination. Testicular pieces were also collected from four conventional boars of the same age (Landrace × Large White × Duroc crossbreed, born at the National Institute of Livestock and Grassland Science). Sperm were collected from epididymides of CryoXeno pigs and frozen using the methods reported by Kikuchi, et al. [14].

2.5. Histologic analysis

Testicular pieces from CryoXeno and conventional pigs were cut into sections of 5-μm thick and stained with hematoxylin and eosin. The seminiferous tubule cross-sections were sorted into the following categories, as described previously: [4,13,15] (1) no germ cells present (tubule cross-sections showing Sertoli cells only); (2) spermatogonia present; (3) spermatocytes present as the most advanced germ cells; (4) round spermatids present as the most advanced germ cells; (5) elongated spermatids present as the most advanced germ cells; and (6) mature sperm (spermatozoa) present in the lumina of the tubules

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