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Generation of Rat Offspring Derived from Sperm Cryopreserved/Banked in the National BioResource Project for the Rat Followed by Transportation to Another Institution

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Abstract: To preserve genetic resources efficiently in rats, sperm cryopreservation is essential. This study aimed to confirm the ability of cryopreserved and transported rat spermatozoa to fertilize through intrauterine insemination. The Komeda miniature rat Ishikawa is a mutant caused by the autosomal recessive mutation *mri*. The epididymal sperm was frozen with egg yolk medium and banked at the National BioResource Project (NBRP), Kyoto University. The sperm was transported to Azabu University, then thawed at 37°C. The thawed semen was inseminated into the uterine horns of recipients; its motility was around 10%. Seven of 15 inseminated female rats became pregnant and 13 live pups were born. The results indicate that rat spermatozoa cryopreserved at NBRP are capable of restoring genetic resources through intrauterine insemination. We also confirmed the usefulness of assisted reproductive technologies for the rat including sperm cryopreservation and intrauterine insemination.

Key words: intrauterine insemination, rat sperm, sperm cryopreservation

Introduction

Here we report a case of the successful generation through intrauterine insemination of rat offspring derived from sperm banked at the National BioResource Project (NBRP) for the rat which was transported to another institution. To preserve genetic resources efficiently, sperm cryopreservation is a very important technology as well as embryo cryopreservation[1, 2]. Applying this technology to banking genetic resources can reduce costs, and prevent infection, diseases and genetic contaminations. Although sperm cryopreservation of the mouse and livestock has been extensively used in genetic resource banking, there have only been two successful reports of rat sperm cryopreservation[3, 4]. The Komeda miniature rat Ishikawa (KMI) is a naturally occurring dwarf mutant caused by an autosomal recessive mutation *mri*, KMI was discovered in a closed colony of Wistar rats, that was established as a segregating inbred colony[5]. Homozygous mutants (*mri/mri*) were born and grew normally until 3-4 weeks old, when they gradually started to develop longitudinal growth retardation without other organ abnormalities. The *mri* mutation was identified as a deletion in the rat gene encoding cGMP-dependent protein

kinase type II (cGKII)[6]. In the present study, epididymal spermatozoa from two KMI males were cryopreserved and used as representatives sperm banked at the NBRP for the rat.

The major goal of the NBRP for the rat in Japan is to facilitate the availability of genetically and phenotypically standardized rat strains for biomedical and life science researches that is available to scientists worldwide. The objective of the present study was to confirm the fertility and efficiency of banked/cryopreserved and transported rat sperm through intrauterine insemination.

Materials and Methods

Animals

Epididymal spermatozoa from two males (n = 2) of the KMI (*mri/mri*) rat were frozen with egg yolk medium supplemented with 0.7% Equex Stm (Nova Chemical Sales, Inc., Scituate, MA, USA) as previously described[2] and banked at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto. The semen donor males were housed individually in standard cages with sawdust as bedding materials. The animal housing room was maintained under standard laboratory conditions (light-dark cycle: 12-12 h, lights on at 06:00 h; temperature: 21 ± 1°C; relative humidity 50 ± 10%). Commercial diets for rats and bottled tap water were available *ad libitum*. The animals were treated according to the Regulations on Animal Experimentation of Kyoto University.

Wistar rats (Slc: Wistar/ST) at the age of 7 weeks were purchased and used as recipients (n = 15) and foster mothers (n = 15). Adult males (Slc: Wistar) at the age of 15-36 weeks that were vasectomized (n = 10) were also used to induce pseudo-pregnancy. They were housed in standard cages with sawdust as bedding materials. For induction of pseudo-pregnancy, the recipient females were housed with the vasectomized males in wire floored cages to make confirmation of mating plugs easy. The animal housing rooms of Azabu University were maintained under standard laboratory conditions (light-dark cycle: 14-10 h, lights on at 06:00 h; tempareture: 21 ± 1°C; relative humidity 50 ± 10%). Commercial rodent diet and bottled tap water were available *ad libitum*. The animals were treated in accordance with Azabu University Animal Care and Use Guideline, and also in accordance with Kyoto University Animal Care and Use Guideline.

Sperm freezing and thawing

The cryopreserved spermatozoa in 0.25-mL straws (IMV Technologies, France) were stored in a dry-shipper contained in a hard shell shipping case (CXR-100; Taylor-Wharton, AL, USA), then transported to the laboratory at Azabu University, Kanagawa. The two straws from different males were thawed in a 37°C water bath for 15 s. Thawed sperm was diluted with 1.0 mL of mR1ECM[7] with 0.4% (w/v) bovine serum albumin (BSA, fraction V; Sigma, St. Louis, MO, USA) at 37°C, then incubated at 37°C in 5% CO_2 in humidified air until insemination. The percentage of motile spermatozoa was visibly assessed and determined by direct observation at 37°C under a light microscope at 100 ×.

Artificial insemination and genotyping of offspring

To confirm the fertility of the cryopreserved and transported sperm, artificial insemination with subsequent development to term was performed. The thawed semen, 100 μL (50 μL; 3-4 × 10^5 sperm cells per mL per each uterus), was surgically inseminated on day-0 into the top of both uterine horns of recipient females that had been mated with vasectomized males. The females received anesthesia with vaporized isoflurane (Escain; Merk, Tokyo, Japan). On the morning of day-22 the inseminated females underwent a Cesarean section to obtain offspring and to confirm the pregnancy and normality of the offspring. Foster mothers that had given birth on day-21 before the Cesarean section nursed the offspring derived from the cryopreserved sperm.

The foster mothers and the offspring were fed and observed until weaning at 3 weeks of birth. At weaning, blood from tail of the offspring was individually recovered to obtain DNA for polymerase chain reaction (PCR) analyses of the genotype. The genotyping was performed by PCR as reported previously[6].

Table 1. Insemination of frozen/transported/thawed sperm derived from the KMI rat * into female Wistar rats

Inseminated sperm (Straw)	No. of inseminated females	No. of delivered females (%)	No. of offspring
A	8	2 (25)	4
B	7	5 (71)	9
Total	15	7 (47)	13

* The Komeda miniature rat Ishikawa.

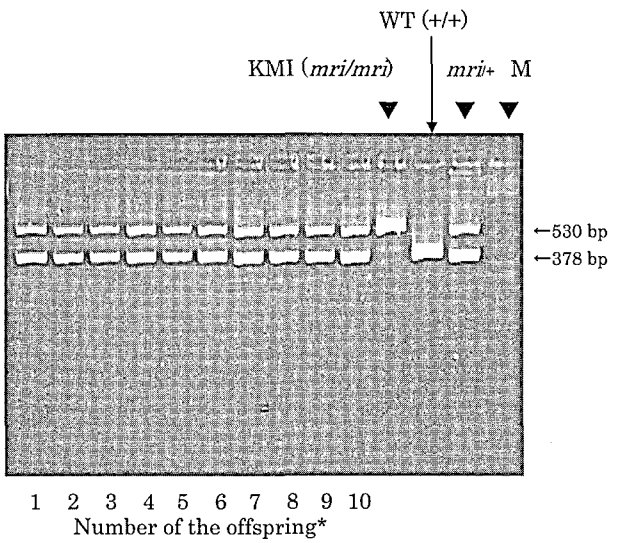

Figure 1. Genotyping of the offspring derived from rat cryopreserved/transported sperm of the Komeda miniature rat Ishikawa (KMI).
*: The offspring was born from females (Wistar) inseminated with frozen/thawed sperm of the KMI, which is a mutant caused by the autosomal recessive mutation *mri*.
M: ϕX174 DNA/Hae III

Results and Discussion

The post-thaw motility of the frozen/transported sperm of the KMI (*mri/mri*) rat was around 10%. Seven of 15 females inseminated with the thawed sperm became pregnant and 13 live pups were obtained from the females (Table 1). At weaning, 3 weeks after birth, 10 of the 13 pups had survived. As shown in Fig. 1, all the offspring has hetero type for the *mri* by PCR analyses.

It was satisfactory from the point of view of banking genetic resources of the rat that we could produce 10 weaned rats derived from the frozen sperm which was transported in two 0.25-mL straws in the present study. To our knowledge, this report is the first successful case of generating rat offspring derived from sperm banked at NBRP and transported to another institution. The introduction of rat genetic materials as cryopreserved sperm instead of living animals into institutions is a useful method because it avoids transportation of live animals, quarantine, and stress. In addition, this method can reduce risk associated with infection and diseases. However, the pregnancy rate and litter size in the present study were lower (47% vs 69% and 1.9 vs 4.6, respectively) than those of our earlier work[2]. We consider that the low number of pups born in spite of the relatively high pregnancy rate in the present study was caused by damage of the sperm during the freezing and thawing procedure as well as boar semen[8]. In the mouse, sperm membrane integrity including the acrosomal membrane is also damaged during the freezing and thawing procedures[9]. In addition, sperm cryopreservation induces sub-lethal damage, reduced activity of the membrane enzyme, injuries to the surface proteins, and diffusion of the membrane proteins[10]. Furthermore, the differences from our early work might also be explained by the small number of males used and the strain difference in the present study. The need for the improvement of the freezing and thawing procedures for rat spermatozoa has been recognized for the efficient generation of offspring from frozen/thawed sperm and in the application of *in vitro* fertilization with frozen/thawed rat sperm.

In summary, the results of the present study indicate that rat spermatozoa cryopreserved at NBRP for the rat in Japan and transported to another institution are capable of restoring genetic resource efficiently through intrauterine

insemination. We also confirmed the usefulness of assisted reproductive technologies for the rat including sperm cryopreservation and intrauterine insemination.

This study was presented in part at the Annual Meeting of the International Embryo Transfer Society, January 2007, Kyoto, Japan[11].

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