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Re-Expression of Rhino-Like Mutated Mouse Phenotype by IVF-ET with Frozen-Thawed Spermatozoa

Hiroshi Imahie[1]*, Taiji Sakata[2], Katsuya Honda[1], Toshihisa Fujiwara[1] and Yuzo Asano[1]

[1]Safety Research Laboratory, Osaka 532 and
[2]Marugo Research Service, Tanabe Seiyaku Co. Ltd., Osaka 532, Japan

Abstract: *Spermatozoa of mutated mice whose appearance quite resembles the rhino mouse were frozen and thawed, then submitted to in vitro fertilization and embryo transfer (IVF-ET). The cryopreservation solution was an 18% raffinose and 3% skim milk mixture, and the spermatozoa were stored in liquid nitrogen at −196°C. Two and half months later, the samples were thawed and fertilized with ICR mouse oocytes in vitro. The motility rate after thawing was approximately 20% and the fertilization rate was 14% (13/95), but 92% (12/13) of the fertilized eggs developed into the blastocyst stage. These twelve embryos were transferred into the uterine horns of a pseudopregnant recipient, and 75% (9/12) were born. The heterozygous F1 offspring did not show signs of the rhino-like phenotype, such as wrinkled skin without hair, but among a total of 39 offspring (F2) derived from brother-sister mating of F1 siblings, 11 (5 males and 6 females) pups showed signs of the rhino-like phenotype, e.g. at two- to three-weeks of age hair fell out, and at 6 months their skin became thick and wrinkled. Histopathological observations showed pilary canal cysts in the cortex of the skin, and dermal cysts were observed in the middle and inner area. These results demonstrate that the gene resources of rhino-like mutated mice can be preserved by the cryopreservation of spermatozoa and re-expressed via IVF-ET.*

Key words: *Cryopreservation, Spermatozoa, IVF-ET, Rhino mouse.*

The rhino mouse, so called because of its characteristic rhinoceros-like skin (hairless and wrinkled), was first described by Howard in 1940 [1]. This characteristic is governed by the autosomal recessive gene which is an allele of the hairless gene, and is located in chromosome 14. Only homozygous mice show the rhinoceros-like phenotype [2–4].

Iwasaki *et al.* happened to find rhino-like mutated mice among black-KK (KK-C) strain [5]. The KK-C strain is characterized as obese and non-insulin dependent diabetes mellitus [6]. According to their report, during the process of brother-sister mating of KK-C, in the fifth generation, mutated mice entirely without hair were found. Loss of hair began at three to four weeks, and by six to seven weeks, all of the hair had fallen out. Successive observations of these animals showed that they came to have sagging skin which folded at about nine weeks, and white spots were formed on the skin. The folded skin and white spots later became remarkable and were outwardly very similar to the rhino mouse. Histopathological characteristics of the skin were same as that of the rhino mouse, namely enlargement of cysts, cornification of the cortex, and panniculitis due to collapse of cysts. Other pathological features in the tissues of major organs were same as those of the KK-C mouse, and enlargement of the islands of Langerhans in the pancreas was also observed.

In this study, we preserved the gene of rhino-like mutated mice also exhibiting the obesity and diabetes syndromes by the method of spermatozoa cryopreservation, and IVF-ET was done with thawed spermatozoa. We studied whether the characteristics of these animals would be maintained in the next generation.

Materials and Methods

Six 14 to 15 week old, rhino-like mutated mice found by Iwasaki *et al.* and maintained at Tanabe Seiyaku Co. Ltd., were used in the present experiment. They were

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*To whom correspondence should be addressed.

hairless. All mice used in this study were bred in a room maintained at 22–24°C, 50–60% relative humidity, and illuminated by artificial light, between 6:30 AM and 6:30 PM.

The methods used in freezing and thawing the spermatozoa were essentially the same as those described by Nakagata and his co-researchers [7–13]. The cryopreservation solution was dissolved in distilled water at 60°C. The solution was centrifuged at 10,000 g for 15 min, the supernatant was filtered and used as the cryoprotectant, and they were stored at −24°C until the experiment. Spermatozoa were collected from caudae epididymidis taken from rhino-like mutated mice. The right and left caudae epididymidis of each animal were minced in 0.3 ml of the cryopreservation solution in the wells of a four-well multi dish, and the spermatozoa were dispersed by shaking the dish at room temperature for about 1 min. A 20 μl suspension was transferred into a sampling tube (volume 0.25 ml) and both sides of the tube were sealed by heating. The tube containing spermatozoa was first cooled with liquid nitrogen gas for 10 min, then plunged directly into liquid nitrogen, where it was stored until thawing. After 2 hr, 7, 10, 74 and 84 days of preservation, samples were thawed as described below, and motility was checked.

The samples were removed from the liquid nitrogen and were thawed in warm water at 30°C for 10 min, then 1 μl of thawed spermatozoa were added to the 200 μl volume of TYH medium. The diluted spermatozoa were placed in an incubator (37°C, 5% CO_2 in air) for 1 hr. Spermatozoa after 74 and 84 days' preservation were used for IVF in this study.

The IVF procedures were essentially those described by Toyoda *et al.* [14]. Female ICR mice (21 weeks old) were each injected with 5 i.u. of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) at an interval of 48 hr. The oocytes were obtained by puncturing the ampullae of oviducts 15–16 hr after hCG injection [15], and introduced into the TYH medium containing the thawed and preincubated spermatozoa mentioned above. At 6 hr after insemination, the oocytes were observed under an inverted microscope, and eggs with two pronuclei and a second polar body were regarded as being fertilized. The embryos which developed into blastocysts were transferred into the uterine horns of one ICR pseudopregnant recipient on day 2.5 p.c. The recipient mouse was mated with a sterility-proven vasectomized ICR male mouse. A vaginal plug was observed after mating, and the animal was considered to be in a state of pseudopregnancy on day 0.5.

The foster mother was allowed to deliver, the F1 pups were mated after becoming adults, and the F2 offspring were also observed. These F2 young were killed at six months, and the mice who had skin like those of the rhino were studied histopathologically after hematoxylin and eosin stain were applied.

Results

At two hr, 7, 10, 74 and 84 days after cryopreservation of spermatozoa, each revival rate was about 20%, and there was no fluctuation in the motility rate depending on the duration of cryopreservation. The fertilization rate with fresh ICR oocytes was 14% (13/95), and 92% (12/13) of the fertilized eggs developed to the blastocyst stage (Table 1). These 12 embryos were transferred into both uterine horns of one pseudopregnant recipient, and the surrogate mother delivered nine offspring (75%) on day 19.5 p.c. (Table 2). The nine newborn were two females and seven males, and coat growth was observed at about one week. The coat color of one male was black and the rest were like wild mice

Table 1. Fertility of the frozen-thawed rhino-like mutated mice spermatozoa assessed by *in vitro* fertilization

No. of oocytes examined	No. of oocytes fertilized (%)	No. of fertilized eggs which developed to blastocyst stage (%)
95	13 (14)	12 (92)

Table 2. Development to live young (F1) of blastocyst embryos produced by *in vitro* fertilization with the cryopreserved rhino-like mutated mice spermatozoa

No. of recipient used	No. of embryos transferred	No. of live young (F1) delivered		
		total (%)	female	male
1	12	9 (75)	2	7

Table 3. Re-expression of rhino-like phenotype in pups (F2) derived from brother-sister mating of heterozygous F1 siblings

No. of live young (F2) delivered	No. of rhino-like mice (F2)		
	total (%)	female	male
39	11 (28)	6	5

with brown hair. These heterozygous rhino-like offspring developed normally, and there was no resemblance to the rhino mouse.

After becoming adults, the F1 offspring were paired by brother-sister mating. As a result, a total 39 offspring (F2) were obtained and in 11 offspring (5 males and 6 females), about one fourth, loss of hair was observed at two to three weeks, and resemblance to the rhino mouse was observed later (Table 3). It was quite difficult to distinguish the rhino-like homozygous mice from heterozygous siblings until two weeks, but in this period loss of hair began from around their eyes, and baldness reached the face, shoulders and abdomen. Finally at ten weeks, all hair became loose, and the sagging, wrinkled skin like that of the rhino mouse was observed.

In the observations of F2 offspring at six months, homozygous rhino-like mice were seen to have wrinkled skin, and they resembled a rhinoceros in appearance (Fig. 1). Histopathological observations showed that the skin became thick and wrinkled. Around the cortex of the skin, pilary canal cysts were observed due to keratonosis, and also in the middle and inner area, follicle-end cysts (dermal cysts) were observed because of the residue of the follicle-end (Fig. 2). Moreover, in dermal cysts, there was also residue of secretion, and the framework of cysts and invasion of inflammatory cells (such as lymph corpuscles, macrophages and so on), were observed in the connective tissue of the corium.

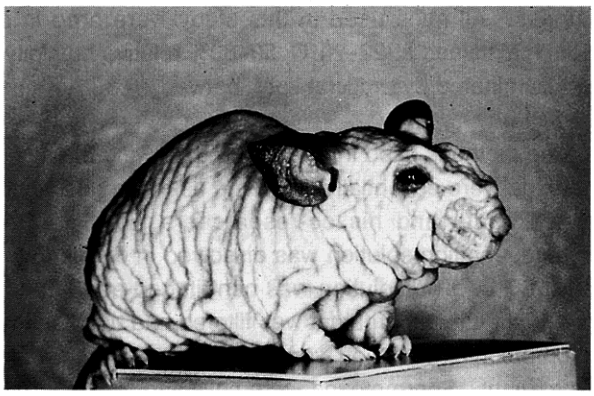

Fig. 1. Male rhino-like mutated mouse of F2 offspring, 6 months old, derived from the KK-C strain. The spermatozoa of rhino-like mutated mice (F0) of the KK-C strain were frozen and thawed, and used for IVF with ICR strain oocytes, The fertilized embryos were transferred to a pseudopregnant recipient, and the surrogate mother delivered heterozygous offspring (F1). They were mated by brother-sister pairing, and homozygous pups (F2) were born. These homozygous offspring (F2) had the same kind of hairless and wrinkled skin as the rhino mouse.

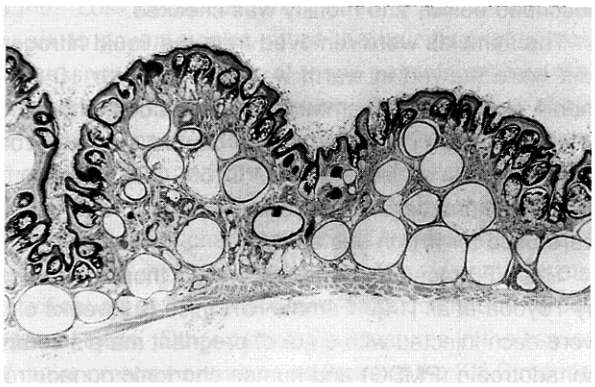

Fig. 2. Photograph of skin of the rhino-like mutated mouse (F2) mentioned in Fig. 1. The surface forms wrinkles and pilary canal cysts in the cortex, and dermal cysts are observed in the middle and inner areas. HE, × 40.

Discussion

Hairless and nude mice are also well known [2]. The skin of hairless mice is controlled by the recessive gene (hr) on chromosome 14, as is the case of the rhino, wrinkles are not remarkable, and the hair comes out again sparsely after falling. In the case of nude mice, their skin is governed by the recessive gene (nu), and there is much more hair than on the rhino. There are no remarkable wrinkles on their face or body. In addition to the hairless and nude mice, some house mice without hair have been reported, but no rhino-like mutated mouse derived from the KK strain characterized by non-insulin dependent diabetes mellitus without hair has been reported.

Among the methods of storing genetic resources of mutated mice, cryopreservation of spermatozoa is a useful one. In mice, this method was introduced recently, and since then it has been applied to artificial insemina-

tion or IVF-ET, and even newborn have been reported [7–13, 16–18].

Nakagata and Takeshima [11] examined the motility and fertilizing ability of frozen-thawed spermatozoa in various strains of mice. In their reports, mouse epididymal spermatozoa from inbred (BALB/c, C3H/He, C57BL/6N, CBA/JN and DBA/2N) and F1 hybrid (B6C3F1, BDF1 and CDF1) strains were compared. Spermatozoa from all of the strains were successfully frozen, although the motility and fertilization rates with ICR fresh oocytes of frozen-thawed spermatozoa varied among strains (motility: 23–62%; fertilization rates: 26–89%). Nearly all 2-cell embryos fertilized by frozen-thawed spermatozoa were transferred to the oviducts of pseudopregnant recipients and 35–62% of embryos developed into normal young.

In our present study, we cryopreserved the spermatozoa of rhino-like mutated KK-C mice, and the thawed spermatozoa were used for IVF with ICR fresh oocytes, after which fertilized embryos were transferred to one recipient. As a result, both the motility and fertilization rate after thawing were low (approximately 20%), but the rate of development to the blastocyst stage was high (92%) and normal young were born at a high rate (75%). Concerning the period of preservation, there were no differences in motility, heterozygous F1 offspring grew normally, and they showed signs of normal reproductive function, and brother-sister mating among heterozygous siblings resulted in homozygous F2 offspring according to the Mendel's Law. Homozygous F2 young had hair loss on the whole body and formed wrinkles, and they resembled the rhino mouse. These results demonstrate that the phenotype of rhino-like mutated mice was preserved and re-expressed via cryopreservation of spermatozoa and IVF-ET.

Nakagata *et al.* [13] reported that the low fertilization rate for low motility frozen-thawed spermatozoa could be improved by partial dissection of the zona pellucida. This assisted fertilization technique may be applied to IVF of the frozen-thawed spermatozoa from rhino-like mutated mice.

Tsubura *et al.* [19] summarized the histopathological characteristics of rhino mice especially on the skin as follows: although the rhino mouse has rudimentary mammary glands, these are thought to be defective due to the impossibility to raise their young. Morphological observations of the female mammary glands of rhino mice were carried out and the results were compared with those of their normal litter mates at various ages. No morphological differences between the rhino mice and their normal litter mates in mammary gland development was seen, and normal secretory changes were observed in the rhino mice after delivery. Furthermore, the sweat glands in the rhino mouse were normal. Anomalies in the rhino mouse therefore appeared to be limited to the skin and pilosebaceous apparatus.

At present, cryopreservation of unfertilized oocytes and fertilized eggs are also proceeding at our facilities. Hereafter, if both male and female zygotes are stored even in the rhino-like mutated mouse, it will be possible to create mice with a variety of genetic backgrounds, including rhino and diabetes mellitus derived from thawed and fertilized zygotes *in vitro*. This seems to be a useful method to create an animal model of diseases.

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