FULL PAPER *Theriogenology*

Production of Transgenic Rabbits Using Centrifuged Pronuclear Zygotes

Masumi HIRABAYASHI[1], Masao HIRAO[2], Ri-ichi TAKAHASHI[1], Ken KIMURA[3], Kazuo HIRASAWA[2], Masatsugu UEDA[1] and Shinichi HOCHI[3]*

[1]*YS New Technology Institute Inc., Ishibashi-Machi, Shimotsuga-Gun, Tochigi 329-0512,* [2]*Ina Research Laboratory, Kitayama Labes Co. Ltd., Ina, Nagano 396-0021,* [3]*Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan*

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ABSTRACT. Superovulation of female rabbits was induced by subcutaneous injection(s) of porcine FSH. Zygotes were recovered 17 to 19 hr after hCG injection and were classified into two categories under a microscope equipped with Nomarski interference-contrast optics at × 200 magnification: (A) zygotes with clearly visible pronuclei, or (B) zygotes with visualized pronuclei after 10 min centrifugation at 12,000 × g. No significant difference between strains was found in the proportion of category-A zygotes (JW 72.6% vs NZW 79.3%). Pronuclei of category-A zygotes were located in the center of the cytoplasm, and the pronuclei of category-B zygotes were slightly moved by centrifugation toward the mass of cytoplasmic lipid droplets. Exogenous DNA solution (5 $\mu g/ml$ of fusion gene composed of bovine αS_1-casein promoter and human growth hormone structural gene) was microinjected into the pronucleus of the JW zygotes. The pronucleus of category-A zygotes with a mean volume of 7.4 p*l* swelled up to 16.6 p*l* (132% increase), while that of category-B zygotes with a mean volume of 6.1 p*l* swelled up to 15.9 p*l* (148% increase). Nevertheless, similar proportions of category-A and category-B zygotes developed into offspring after transfer to recipient females (11.1 and 11.2%, respectively). The efficiency to produce hGH-carrying transgenic rabbits was 0.9% (2/235) from category-A zygotes and 0.5% (1/215) from category-B zygotes (P>0.05). To date, transgenic rabbits have been produced without centrifugation of pronuclear zygotes. However approximately 25% of fertilized rabbit zygotes can be used for DNA microinjection after they have been centrifuged to visualize their pronuclei.

KEY WORDS: centrifugation, cytoplasmic lipid, hGH, pronucleus, transgenic rabbit.

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Transgenic technology is being applied not only to small experimental rodents such as mice [4, 13] and rats [17, 26] but also to large domestic species such as goats [10], sheep [7], pigs [33] and cattle [21]. The most conventional and reliable method to produce transgenic animals is the pronuclear microinjection of exogenous DNA. Due to the differences in the volumes of cytoplasmic lipid droplets among species, pronuclear zygotes from some species, including sheep, pigs and cattle, have to be centrifuged before DNA microinjection in order to visualize their pronuclei. The high magnitude g force on the pronuclear zygote itself was reported not to be detrimental to the subsequent developmental potential in cattle [31].

The first successful production of transgenic rabbits was reported in 1985 independently by Brem *et al.* [3] and Hammer *et al.* [14]. The specific advantage of using transgenic rabbits would be their availability for research on lipid metabolism using hepatic lipase [11] and on arteriosclerosis human apolipoprotein A-1 [8], and for protein production such as human interleukin-2 [5], insulin-like growth factor-1 [35] and erythropoietin [6]. There are more than 50 reports referring to transgenic rabbits. To produce these transgenic rabbits, pronuclear zygotes from superovulated or spontaneously ovulated females have been used without centrifugation. Zygotes without visible pronuclei have been discarded because they were considered as unfertilized eggs or fertilized zygotes but not at a pronuclear-stage.

* CORRESPONDENCE TO: Dr. HOCHI, S., Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3–15–1 Tokida, Ueda, Nagano 386–8567, Japan.

In this report, it is indicated that approximately 25% of pronuclear-stage zygotes can be used for the production of transgenic rabbits after they have been centrifuged to visualize their pronuclei before DNA microinjection. The efficiency of producing transgenic rabbits from centrifuged zygotes was comparable with that from non-centrifuged zygotes.

MATERIALS AND METHODS

Experimental design: In the first series of experiments, the number of rabbit zygotes with clearly visible pronuclei and those with pronuclei visualized after high magnitude centrifugation was counted from Japanese White (JW) and New Zealand White (NZW) rabbits (n=8 each). Two different protocols were used to induce superovulation in these rabbits (4 JW + 4 NZW each). In the second series of experiments, pronuclear zygotes from the JW strain were classified into the two categories as mentioned above, and exogenous DNA solution was injected into their pronucleus. Then, the volume change in the pronucleus following DNA microinjection was compared between the two categories. By surgical transfer and PCR analysis, the efficiency of producing transgenic rabbit offspring was examined.

Egg recovery: Specific-pathogen-free/virus antibody-free Kbs:JW rabbits (3.5 to 5.3 kg) and Kbs:NZW rabbits (3.5 to 4.9 kg) were housed in an environmentally controlled room with a 12-hr dark:12-hr light cycle at a temperature of 23 ± 3°C and humidity of 60 ± 15%. They were given free access to pelleted rabbit chow (Oriental Yeast Co., Japan) and fil-

tered water. The female rabbits were superovulated either by subcutaneous injections of 0.5 AU porcine follicle-stimulating hormone (pFSH; Denka Pharmaceuticals Inc., Japan) dissolved in 0.9% saline every 12 hr over 3 days or by a single injection of 3.0 AU pFSH dissolved in 10% polyvinylpyrrolidone (PVP; Wako Pure Chemical Industries., Japan), as described previously [15]. The females were then subcutaneously given 75 IU human chorionic gonadotropin (hCG; Teikoku Zouki Co., Japan) 72 hr after the first injection of pFSH and were mated with fertile male Kbs:JW rabbits. The oviduct ampullae were flushed with EmbryoTec medium (Nihon Zenyaku Co., Japan) 17 to 19 hr after hCG injection, and the eggs recovered were kept in TCM199 (Gibco BRL, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Equitech Bio, TX, U.S.A.) at 37°C in 5% CO_2 in air.

Classification of pronuclear zygotes: There were two types of rabbit eggs at recovery: zygotes with clearly visible pronuclei and eggs with granulated cytoplasmic lipids, as shown in Fig. 1. The former zygotes were classified as category-A. The latter granular eggs were centrifuged for 10 min at 12,000 × g in 1 m*l* of Dulbecco's phosphate buffered saline (D-PBS; Gibco BRL) + 20% FBS, as reported previously [20]. Zygotes with visible pronuclei after the centrifugation were classified as category-B. For this classification, a microscope (TMD-300; Nikon, Japan) equipped with Nomarski interference-contrast optics was used at × 200 magnification.

DNA construct: A recombinant DNA construct containing the hGH gene was prepared as previously described by Ninomiya *et al.* [26]. Briefly, the *Hin*dIII-*Bam*HI fragments of the 5' regulatory region of the bovine αS_1-casein gene prepared by PCR were inserted into the *Bam*HI sites of plasmid p0GH containing the coding regions, introns, and poly-A signal of hGH (Nicholas Institute Diagnostics, CA, U.S.A.). The fusion gene was excised by *Hin*dIII and *Xba*I, separated by 1.2% agarose gel electrophoresis, and extracted from the gel with Geneclean II (BIO101, CA, U.S.A.). For microinjection, the DNA (2.8 kb) was dissolved in 10 mM Tris-HCl (pH7.6)/0.1 mM EDTA to yield a final concentration of 5 μg/m*l* and stored at –20°C until use.

Microinjection: The DNA solution was injected into the male pronucleus of JW rabbit zygotes in D-PBS + 20% FBS with the aid of a pair of micromanipulators (MO-102 and MO-103; Narishige Scientific Instrument Laboratory, Japan) at × 200 magnification, as shown in Fig. 2. The volume of the pronucleus was calculated from the microphotographs taken before and after DNA microinjection. To determine the effect of centrifugation on the pronucleus volume, some category-A zygotes were centrifuged and the volume of the pronucleus was calculated as well. Morphological survival of zygotes was assessed 1 hr after DNA microinjection. The surviving zygotes with a small amount of TCM199 + 10% FBS were transferred to the oviduct ampullae of the Kbs:JW female recipients (3.4 to 4.6 kg) that had received 75 IU hCG on the preceding day (maximum 40 zygotes per recipient).

PCR analysis: Offspring were recovered from recipient rabbits on Day-28 of the gestation (Day-1 was defined as the day of surgical transfer). The presence or absence of the fusion gene was examined in the tail tissue of the offspring by PCR, as described previously [26]. The primer pairs used for amplification of the transgene were HGH003 ($_{5'OH}$ ACACCTACCAGGAGTTTGTAAGCTC $_{3'OH}$) and HGH103 ($_{5'OH}$ CTGGAGACCAGCTCCCATTGTTACT $_{3'OH}$).

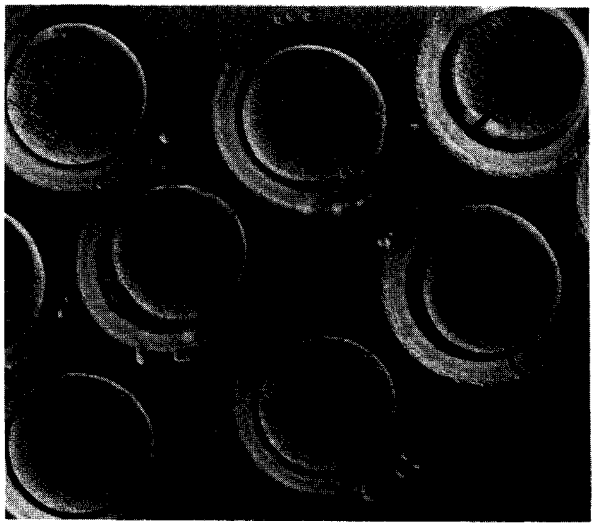

Fig. 1. Heterologous population of rabbit zygotes recovered 17–19 hr after hCG injection. Zygotes with clearly visible pronuclei (arrows), and zygotes darkly granulated by the cytoplasmic components. Bar=100 μm.

Statistics: Three to five experiments were replicated in each series. Data were analyzed by one-way analysis of variance (ANOVA). Percentage data were arcsin transformed before being subjected to ANOVA. The significant differences were determined by Fisher's protected least significant difference test using STATVIEW (Abacus Concepts, CA, U.S.A.). A value of $P<0.05$ was chosen as an indication of statistical significance.

RESULTS

The mean proportion of category-A zygotes among the total pronuclear zygotes was compared between JW and NZW rabbits (Table 1), and there was no significant difference. There was a large variation in the proportion of category-A zygotes among individual donors. Three of 8 NZW donors provided only category-A zygotes, while all zygotes from 1 NZW donor were classified as category-B. The superovulatory regimen was not the factor influencing the proportion of category-A zygotes, although it did affect the recovery rate of the pronuclear zygotes. Ovarian response to gonadotropin stimuli seemed to be related to the proportion of category-A zygotes when the 16 donors were divided in half (8 each) based on the number of ovulation points in each donor, but the difference of the proportion was not significant ($P=0.11$).

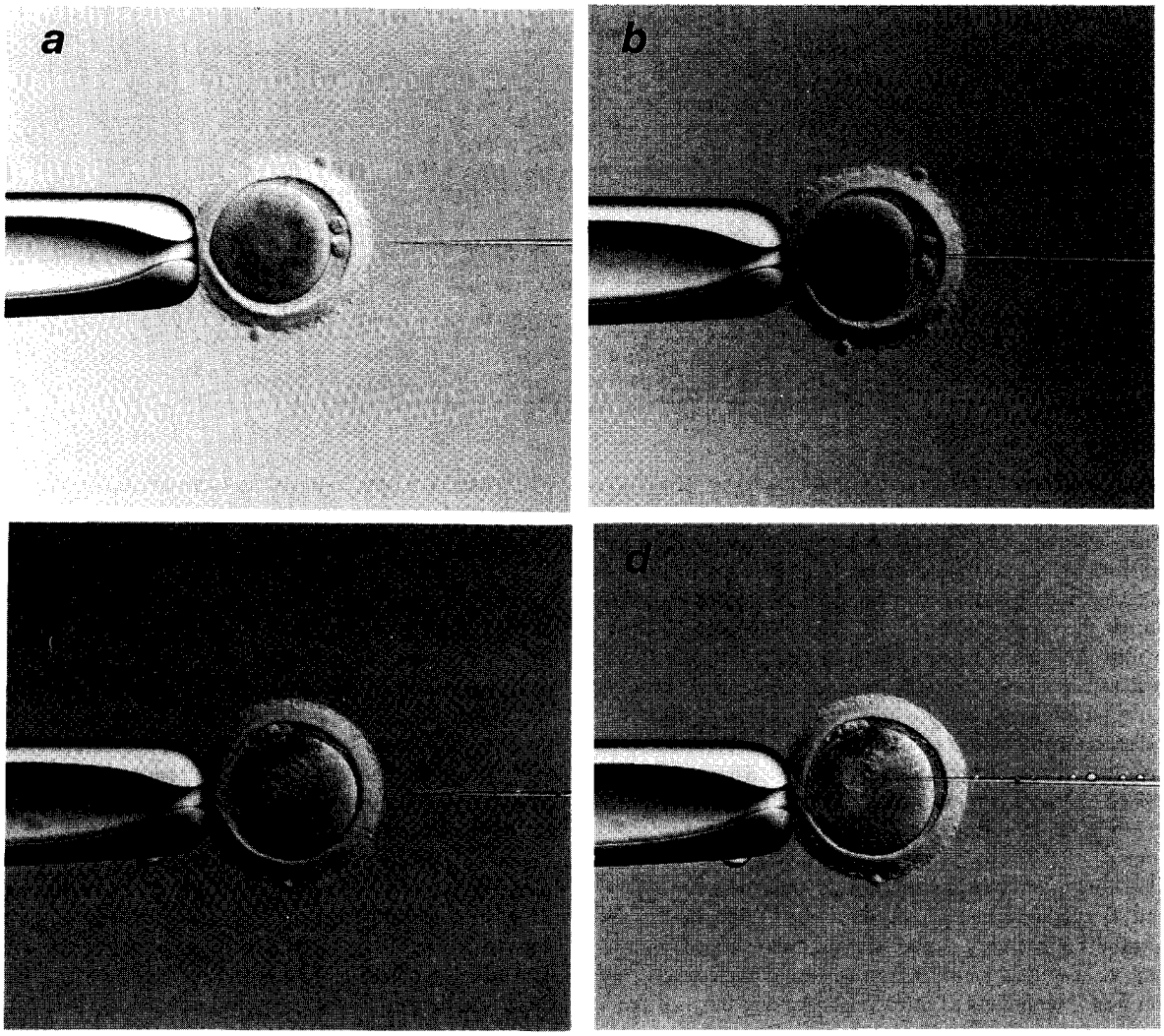

Fig. 2. DNA microinjection into pronucleus visible without centrifugation (a: before injection, b: after injection) and into pronucleus visualized by centrifugation (c: before injection, d: after injection).

Table 1. Effects of rabbit strains, FSH treatments, and superovulatory responses on the recovery of zygotes with clearly visible pronuclei

	No.(%) of pronuclear zygotes			No. of donors			
		Visible pronuclei	Visualized pronuclei after centrifugation		[A]/[A]+[B] ratio		
Factors	Total	[A]	[B]	=0	0<r≤0.5	0.5<r<1	=1
Strains							
JW (n=8)	219	159 (72.6)	60 (27.4)	0	2	6	0
NZW (n=8)	169	134 (79.3)	35 (20.7)	1	0	4	3
Superovulation							
FSH/PVP × 1 (n=8)	118	82 (69.4)	36 (30.6)	1	1	4	2
FSH/Saline × 6 (n=8)	270	211 (78.1)	59 (21.9)	0	1	6	1
Ovulation points							
≤33 points/donor (n=8)	119	100 (84.0)	19 (16.0)	0	1	4	3
>33 points/donor (n=8)	269	193 (71.7)	76 (28.3)	1	1	6	0

Table 2. Volume changes in JW rabbit pronuclei in the non-centrifuged and centrifuged zygotes during DNA microinjection

Centrifugation	No. of zygotes evaluated	Volume of male pronucleus: mean ± SD: p*l*		
		Initial	Post-injection	(% increase)
–	20	7.4 ± 3.3	16.6 ± 8.0	(132 ± 94)
+	14	6.1 ± 3.3	15.9 ± 10.7	(148 ± 70)

Table 3. Effects of centrifugation of zygotes on the production of transgenic rabbits

Centrifugation	No. (%) of zygotes		No. of pregnant recipients	No. (%) of offspring	
	Injected	Surviving		Born	Transgenic
–	235	226 (96.2)	5/7	26 (11.1)	2 (0.9)
+	215	208 (96.7)	5/7	24 (11.2)	1 (0.5)

The pronuclei of category-A zygotes were located in the center of the cytoplasm, and the pronuclei of category-B zygotes were slightly moved by centrifugation toward the mass of cytoplasmic lipid droplets (Fig. 2). The mean volume of the male pronucleus in category-A zygotes tended to be larger than that in category-B zygotes (Table 2). However, this difference was not due to the effect of centrifugation itself, because centrifugation of category-A zygotes (n=9) resulted in no decrease in the pronucleus volumes (8.0 p*l* before centrifugation vs. 9.0 p*l* after centrifugation). After DNA microinjection, the volume of the pronucleus increased 132% (2.32 times original volume) for category-A zygotes and 148% (2.48 times original volume) for category-B zygotes.

One hour after DNA microinjection, most of the category-A and -B zygotes seemed to be surviving (Table 3). Similar proportions of category-A and -B zygotes developed into offspring, as 26 and 24 viable offspring were obtained from 5 of 7 and 5 of 7 recipients, respectively. Among them, 2 and 1 offspring derived from category-A and -B zygotes, respectively, all of which were male, were found to be the PCR-positive transgenic rabbits. The overall efficiency to produce hGH-carrying transgenic rabbits was 0.9% of category-A zygotes and 0.5% of category-B zygotes (P>0.05).

DISCUSSION

The rabbit zygotes classified in category-B appeared to be granulous due to the cytoplasmic lipid droplets. The early cleavage-stage porcine and bovine embryos contain a large amount of lipid droplets in the cytoplasm, which have a close spatial relationship with smooth endoplasmic reticulum [19]. The cytoplasmic lipid droplets are thought to play a role not only in providing a source of nutrients to the cells but also in modifying the physical properties and functions of the plasma membranes [30]. Abe *et al.* [2] reported that the number and size of lipid droplets in the cytoplasm of *in vitro*-produced bovine embryos were influenced by the presence of serum in the culture medium. Estrogen, one of the major serum components, may be involved in the deposition of cytoplasmic lipid droplets [12]. During the last stage of oogenesis, oocytes exposed to a higher concentration of estrogen for a longer period in the follicular fluid may store an abundance of lipids in their cytoplasm.

It was shown that centrifugation of porcine and bovine zygotes at 15,000 × g for 3 to 5 min resulted in stratification of the cytoplasm that renders the pronuclei visible under differential interference contrast microscopy, without detectable loss of the viability [31, 32]. However, centrifuged sheep zygotes were reported to have a lower potential for development into blastocysts than non-centrifuged zygotes [25]. Transfer of centrifuged mouse zygotes to pseudopregnant recipients also resulted in a decrease in the number of newborn offspring [23, 24]. In the present study, rabbit zygotes classified into category-A and -B developed into viable offspring at similar rates (11.0 and 11.2%, respectively), suggesting the presence of a species difference in the tolerance of pronuclear zygotes to high magnitude centrifugation. The developmental potential of DNA-injected zygotes has been reported to be considerably reduced in mice [4], rats [16] and cattle [20. 27]. Because our recent study indicated that 37% of intact rabbit zygotes after transfer could develop into offspring [15], a remarkable loss of developmental potential in DNA-injected rabbit zygotes occurred in the present study.

In the present study, the overall efficiency of producing transgenic rabbits per injected zygote was 0.7%, which was within the range of 0.3 to 2.5% reported to date [1, 9, 18, 22, 28, 29]. Although the sample size of transgenic rabbits (n=3) was small, all founders derived from non-centrifuged and centrifuged zygotes had the potential to express the hGH transgenes (analysis by RT-PCR; data not shown). From a practical point of view in the production of transgenic rabbits, the use of centrifuged pronuclear zygotes, as well as the development of more effective protocols for inducing superovulation and for cryopreserving pronuclear zygotes, may be important, because the cost of obtaining an adequate amount of rabbit zygotes is high.

In conclusion, approximately 25% of fertilized rabbit zygotes could be used for DNA microinjection after they had been centrifuged in order to visualize their pronuclei. The centrifuged zygotes were possible sources available for the

production of transgenic rabbits.

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