IN VIVO DEVELOPMENT AND MICROINJECTION OF RABBIT ZYGOTES

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Summary

Sixty-one 5-11 month-old California, Chinchilla and New Zealand White rabbit were employed in this investigation. Thiry-three does were superovulated by injecting FSH/HCG subcutaneously or intravenously and then sacrificed at different hours after mating. The ova were collected from the fallopian tubes with Ham's F-10 medium supplemented with 0.4% bovine serum albumin (BSA) and 1% pregnant rabbit serum (PRS). Embryos were examined under an inverted DIC microscopy for observing the stage of development. We have found that the fertilized ova formed pronuclei at 19 - 20 hr postcoitus. Approximately at 26, 64 - 78 and 84 - 88 hr after mating, the fertilized ova cleaved further to 2-cell, morulae and blastocyst stage respectively. Another 28 does were allocated to the gene transfer study. Fourteen of the 28 does were sacrificed at 19 - 20 hr to donate the pronuclear stage ova for gene injection. The other 14 does were induced to pseudopregnancy by injection of 100 IU HCG intravenous as recipients. Four hundreds and seventeen ova were injected totally and 212 gene injected zygotes were transferred into the recipient oviducts. Five recipients became pregnant and 10 fetuses were obtained. Eight of the 10 fetuses were analysed for gene incorporation, but none of them were transgenic.

(**Key Words**: Microinjection, In Vivo Development, Rabbit Zygotes)

Introduction

The microinjection technique was thought to be the most widely used method of gene transfer in mammalian zygotes (Primrose, 1987; Whitelaw and Clark, 1989; Clark et al., 1989). Although there is still a limited success in the control of gene expression, recently, transgenic animals have been produced by this method in many species, such as mouse (Brinster, 1981; DePamphilis et al., 1988; Harbers et al., 1981; Walton et al., 1987), rabbit (Buhler et al.,1990; Hammer et al., 1985; Knight et al., 1988), sheep (Clark et al., 1989) and pig (Hammer et al., 1985; Hammer et al., 1986). Even the transgenic bovine fetuses have been reported (Biery et al., 1988). The objectives of this study are firstly, to confirm the timing of pronuclei formation and subsequent in vivo development of fertilized rabbit ova. Secondly, we attempt to set up the microinjection technique of rabbit zygotes so as to apply this model system to the large domestic species.

Materials and Methods

I. Preimplantation development of embryos.

Thirty-three 5-11 month-old California, Chinchilla and New Zealand White rabbits were treated with FSH/HCG to superovulate and were sacrificed at various hours postcoitus. The embryos were recovered by flushing the excised oviducts and uteri with Ham's F-10 medium supplemented with 0.4% bovine serum albumin (BSA) and 1% pregnant rabbit serum (PRS) as described by Cheng et al. (1988). Embryos were examined under an inverted DIC microscopy to identify the formation of pronuclei and evaluate their developmental stage in vivo.

II. Injecting the procine growth hormone (pGH) gene into pronucleus of zygotes

A. Preparation of the pGH gene

The porcine growth hormone (pGH) structural gene was cloned from a porcine genomic library (K. B. Choo, unpublished data). The pGH gene fragment was excised from the clone and electrophoretically separated from the cloning vector.

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Received August 20, 1990
Accepted December 18, 1990

The DNA fragments then were recovered from the agarose gel by electro-elution (Maniatis et al., 1982).

B. Animal treatment

Fourteen 5-8 month-old donors were super-ovulated and mated by the method as previously described by Cheng et al. (1988). According to the results of table 1, the donors were sacrificed at 19 - 20 hr after mating for collecting the pronucleus stage ova. The time of recipient does that induced to pseudopregnancy with HCG injection was 4-6 hr later than the donors.

C. Manipulation and transfer of ova.

A total of 465 pronuclear stage ova were collected in this study. Eighteen were used as control and 30, 29 and 384 ova were allocated to the needle penetration, T. E. (10mM Tris-Cl, 0.1mM EOTA, pH = 7.4) buffer injection and pGH gene injection group, respectively. The pronuclei of needle penetration ova were not injected with fluid but punctured with a blind end microneedle which tip was less than 1 μm in outer diameter (OD). Both in the buffer and gene injection groups, 2-5 pl solution was injected into the male pronucleus of zygotes (figure 2). Whereas the tip of injection micropipette were circa 1-2 μm in outer diameter. Control and manipulated ova were both cultured in an incubator maintaining at 38 ℃ and 5% CO_2 in humidified air for evaluating their further development. Only the gene-injected ova were transferred into the fallopian tubes of 14 recipient does after 1 hr incubation by laparotomy.

D. Analysis of gene integration

Six pups that generated from the gene injected zygotes were sampled by incising their ear or tail tissues for extracting genomic DNA. The extracted DNA was digested by the restriction endonuclease Pst I completely, and then hybridized to the DNA probe after transferring from the gel to nitrocellulose (N. C. paper) by Southern blotting technique. The DNA probe was prepared from the same DNA fragments used for microinjection. The DNA was ^{32}p-labelled by the nick translation procedure (Maniatis et al., 1982).

Results and Discussion

I. Pronuclei formation and their subsequent development of fertilized ova

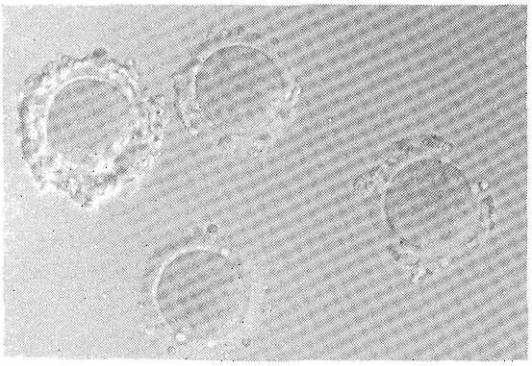
Eighty-one does were sacrificed to investigate the cell stage of zygote development in vivo. The male and female pronuclei of fertilized ova are formed and visualized at 19-20 hr postcoitus which is the time most readily for nuclear injection (table 1; figure 1 a). Twenty-six hours after mating, the zygotes began to cleave (figure 1 b). Up to 72-78 hr and 84 hr onward, most of the embryos developed into morulae and blastocyst stage respectively (table 2; figure 1 c & figure 1 d).

In Taiwan, the ambient temperature in summer is too high to perform normally in physiological status for most domestic species under an opening feeding system. In the meanwhile, the quality and

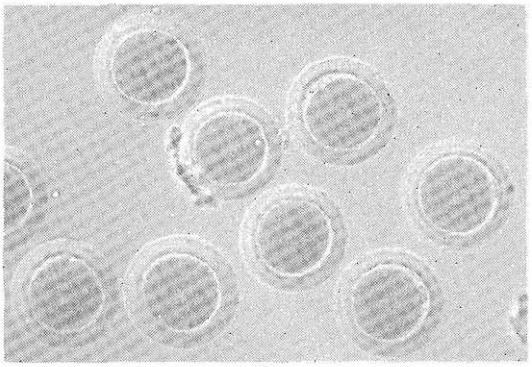
TABLE 1. THE MORPHOLOGY AND CHARACTERISTICS OF RABBIT ZYGOTES AT VARIOUS HOURS POSTCOITUS

Hours postmating	Heads of animal	Morphology and characteristics of zygotes
15-17	2	Unfertilized; the ova are surrounded by thick layers of cumulus cells.
18	2	Some of the ova are not fertilized yet and coated by cumulus cells. Pronuclei are usually unvisible
19	9(7)*	A few eggs cohesive with a little cumulus cells; pronuclei of most fertilized ova are visible.
20	7(7)	Pronuclei of the fertilized ova are clearly visible with no cumulus cells on the surface of zona
23	2(1)	No cumulus cells attached to the zona pellucida of zygotes; some pronuclei still exist.

*Numbers in the parentheses are heads of does that pronuclei being observed.



(a)



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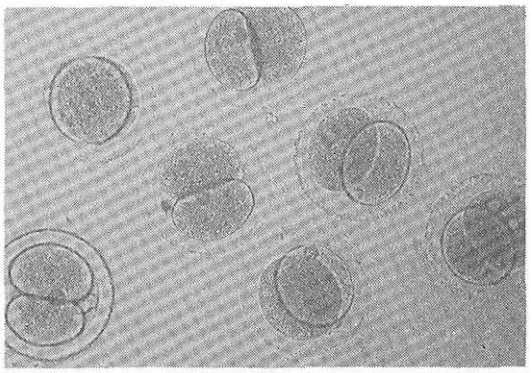

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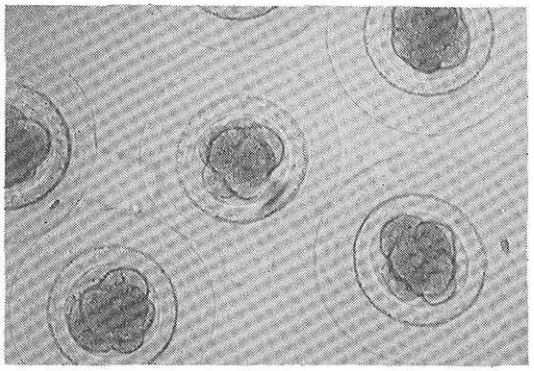

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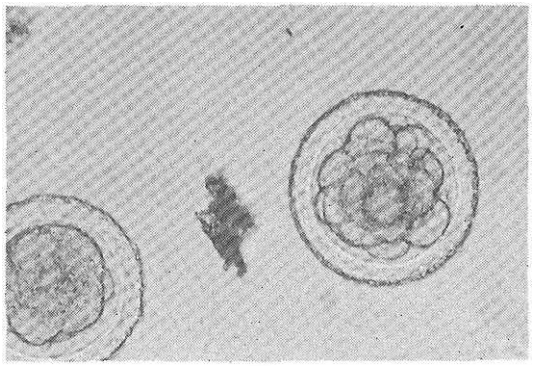

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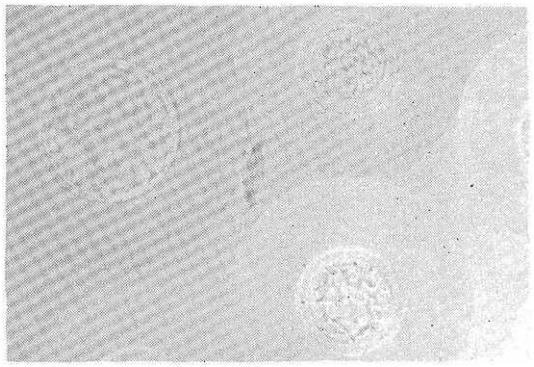

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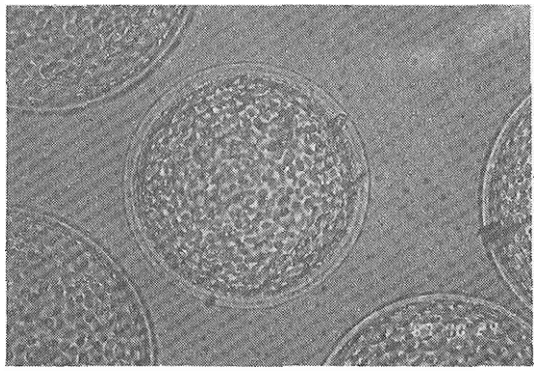


(g)

Figure 1. In vivo development of rabbit zygotes.

a. Ova recovered at 18 hr after mating. Some cumulus cells still attached to the surface of zona pellucida (150 X).
b. Fertilized rabbit ova collected at 19.5 hr postcoitus. The male and female pronuclei are clearly formed (150 X).
c. Two-cell stage embryos collected at 26 hr postcoitus. Note the mucin coat was still thin (200 X).
d. Six to 8-cell stage embryos recovered during

36-40 hr postcoitus (150 X).

e. Sixteen-cell to morulae stage embroys recovered at 54 hr postcoitus (300 X).

f. Blastocysts recovered at 84 hr after mating showing various degrees of development in vivo. The mucin surrounding zona pellucida was quite prominent (100 X).

g. The expanded blastocysts were recovered at 108 hr postcoitus (100 X).

morphology of the embryos was deteriorated when the superovulated dose was administered to the animals (Champlin et al., 1987; Evans and Armstrong, 1984; Fujimoto et al., 1974). In this observation, we have found the similar resultants especially for the blastocyst stage embryos. A large proportion of superovulated blastocysts showed a shrinking or collapsing blastocoel right after being recovered with flushing medium (Ju

TABLE 2. THE IN VIVC DEVELOPMENT OF RABBIT EMBRYOS AT VARIOUS TIME COURSES

Hours after mating	Heads of animal	Number of embryos	Ratios of various cell stages
26	13	132	1 - 2 - cell stage
28-30	5	38	2 : 4-cell = 4 : 34
38-48	8	71	8 : 16-cell = 15 : 56
54-64	5	88	16~morulae stage
72-78	3	14	M : B = 13 : 1
84-88	5	45	M : eb : B : EB = 8 : 7 : 29 : 1
96-108	7	50	B : EB = 34 : 16
136-144	2	14	B : EB = 4 : 10

M : morulae
B : blastocyst
eb : early blastocyst
EB : expanded blastocyst

et al., unpublished data.)

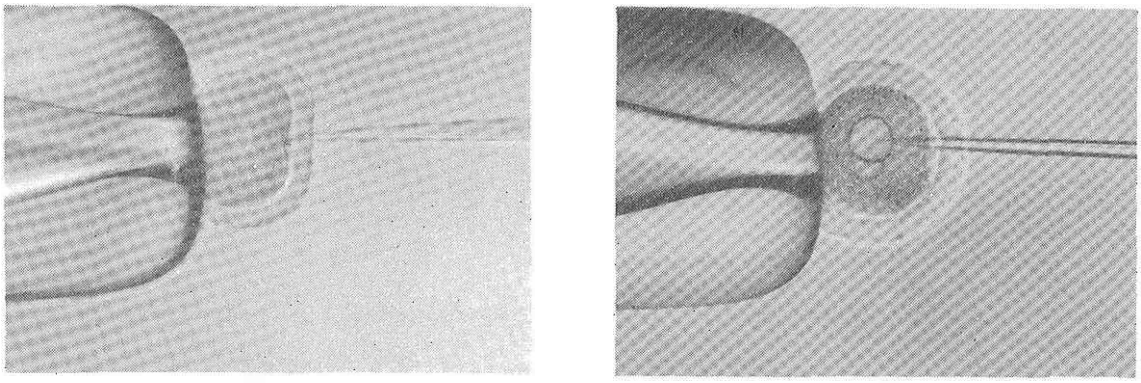
Ⅱ. The development of manipulated zygotes in vitro and in vivo

In the needle penetration group, 10% of the punctured ova lysed or blocked in division after culture in vivo and 66.7% ova developed into morulae stage as well as the control group. While in the gene and buffer solution injection groups, there were 34% (pGH gene) and 62.1% (T. E. buffer) of the injected zygotes suffered lysis or block in subsequent cultivation, which were higher than that of the control and needle penetration groups (table 3). Only the gene injected zygotes were transfered into the fallopian tubes of pseudopregnant does. Five of 14 recipients became pregnancy and 10 fetuses were obtained. Of the 10 fetuses, one had aborted at day 19 after mating, the other 9 had grown to term and delivered normally. Unfortunately, three of the 9 youngs were bitten to death by the foster mother right after being birth.

The possible explanation for the low viability of manipulated zygotes developed in vitro and in vivo were mainly caused by the larger tip size of injection micropipettes and by introducing the foreign materials into the pronuclei of zygotes. When the smaller microneedle (OD $\leq$ 1 μm) were used, the percentage of zygotes developing into morulae stage were 66.7% which were significantly higher than the T. E. buffer injection group (3.4%; OD $\doteq$ 1-2 μm, $p < 0.05$). In the gene injection group, the OD of micropipettes are about 1-2 μm, which are larger than the injection pipette of mouse and sheep eggs described by Walton et al. (1987) and DePamphilis et al. (1988). Again, the larger needle size accompanied with the injection of foreign DNA solution, is supposed to be the most important factor that resulted such a low percentages (5%) of offsprings produced in this study.

Ⅲ. Analysis of gene integration

Eight of the 10 fetuses were sampled to detect



(a) (b)

Figure 2. Injecting the pronucleus of fertilized ova.

a. Penetrating an ovum by microneedle just before injection (300 X).

b. The ovum have been injected with gene solution. Note that swollen male pronucleus (300 X).

TABLE 3. THE VIABILITY AND DEVELOPMENT OF MICROMANIPULATED RABBIT ZYGOTES CULTURED IN VITRO OR TRANSFERRED INTO THE RECIPIENTS

Treatments	Control		Injection groups[2]	
	Untreated	Penetration[1]	TE buffer	pGH1 gene
No. zygote injected	18	30	29	388
Lysed/blocked in development	5.6%	10%	62.1%	33.7%
Premorulae stage	16.7%	23.3%	34.5%	–
Morulae stage	77.8%	66.7%	3.4%	–
No. ova transfered/ No. recipients	–	–	–	212/14
No. pups/No. litters	–	–	–	10/5[3]

[1] The outer diameter (OD) of the tip of penetration needles are < 1 μm.

[2] The OD of injection pipettes are 1-2 μm (injection volume ca. 2-5 pl).

[3] One of the 10 fetuses was aborted at the 19th day after pregnancy, three gene injected offsprings were bitten to death by the foster mother, and only 6 pups were survived. Two of the six have not been analysed for gene integration yet.

the integration of the pGH gene. Although none were pGH gene integrated, three of the four matured rabbits die at 176, 208 and 371 days of age, respectively. One showed the hind legs paralysis and the other 2 did not show significant clinical syndrome. The reasons are quite obscure for the analysis of mosaicism of pGH gene in other organs or tissues have not been performed so far.

According to Hammer et al. (1985, 1986), the incorporation rate of the foreign gene into the genome of embryos is about 13% in rabbit, which is higher than for sheep (1.3%) and pig (10.4%) but lower than for mouse (10-25%; Brinster et al., 1981; Harbers et al., 1981; Palmiter and Brinster, 1985). The success rate of gene incorporation is probably involved in the injection volume, concentration of gene solution and forms of gene segment to be injected (Simons et al., 1988). We have not succeeded in producing a transgenic rabbit in this study. To promote the efficiency of intrducing the foreign gene into the genome of embryos, we at least have to minimized the damage of injection ova by improving the tip size of the injection micropipettes.

Acknowledgements

This project was supported by a grant NSC 78-0409-B005-03 from the National Science Council, Republic of China. We also give thanks to the Agricultural Biotechnology Laboratories for the great help in gene analysis.

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