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Factors Affecting the Efficiency of Introducing Foreign DNA into Rabbit Zygotes by Cytoplasmic Injection

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Abstract: Cytoplasmic injection of exogenous DNA was a high potential methodology that would tremendously increase the efficiency of transgene. In the present study, researchers take a new look at the ancient method and investigate the influencing factors on embryonic development and transgenic efficiency following cytoplasmic injection using pEGFP-N1 plasmid. In the first experiment, researchers evaluated embryonic development and EGFP expression in embryos produced by cytoplasmic injection with different concentrations of pEGFP-N1 plasmid (30, 45, 60 and 75 ng μL^{-1}) and found that the concentration of 60 ng μL^{-1} was optimal for increasing in both blastocysts rate and EGFP-positive blastocysts rate. In the second experiment, the embryonic development and EGFP expression after cytoplasmic injection with different volumes (40, 50 and 60 pL) of exogenous DNA was investigated, the result showed that the optimal volume of injected was 40 pL with which approximately 54.5% of the blastocysts rate and 59.3% of the blastocysts expression EGFP were obtained. Finally, in order to find out the most appropriate timing for cytoplasmic injection, three time periods (16, 18 and 20 h) were assayed to harvest the zygotes. After being injected and cultured, the result confirmed that 16 h group (68.6%) had a significant differences compare to 18 h group (44.9%) and 20 h group (9.7%) at EGFP-positive blastocyst rate but there was no significant differences as to blastocyst rate among these three groups. Two pups were given birth after embryo transplantation. The integration of the foreign DNA was confirmed by PCR and an expected 720 bp products showed that both of the two pup's tissues including kidneys and livers carry *EGFP* gene. The results of frozen tissue sections analyzed by confocal laser scanning microscope and western blot also were confirmed that EGFP expression in kidney and liver. In conclusion, the results of this study imply that the method of cytoplasmic injection at one-cell stage is feasible for producing transgenic animals which can significantly improve the transgenic efficiency.

Key words: Rabbit zygote, cytoplasmic injection, transgenic efficiency, pEGFP-N1, animals

INTRODUCTION

Since, the first transgenic mouse was born at Yale University produced by Gordon and his associates (Gordon *et al*., 1980), transgenic technique has developed so fast during the past decades and various of transgenic animals had obtained including cows (Salamone *et al*., 2006), rat (Swift *et al*., 1984), mouse (Gordon and Ruddle, 1983), pig (Naruse *et al*., 2005), goat (Ebert *et al*., 1991), rabbit (Strojek *et al*., 1987), rhesus monkeys (Niu *et al*., 2010), etc. Correspondently, several methods are developed for generation of transgenic animal including direct microinjection of foreign DNA into the pronucleus of fertilized eggs, nuclear transfer using genetically modified donor cells, viral-based constructs for the delivery of exogenous DNA into embryos and sperm cells as transgene vectors. However, these techniques are still relatively inefficient, technically demanding, costly and impractical in most other animal species. Thus, the challenge is to find a high efficiency and steady technique

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for producing transgenic animals. In the early days, cytoplasmic injection of exogenous DNA as a less frequently used method has been extremely inefficient compared to others methods. The manipulation of the method involves inserting a glass injection pipet through the zona pellucida and into the embryonic cytosol where the exogenous DNA is injected. Brinster conducted a comparative study between pronuclear and cytoplasmic injection and found that pronuclear injection of linear DNA molecules resulted in an integration efficiency of 25-30% while only 0.89% by cytoplasmic injection (Brinster *et al*., 1985). In order to increase the transgenic efficiency, some improved measures had been chosen. Page *et al*. (1995a, b) point out that about 12.8% of the pups born from zygotes cytoplasmically microinjected with a polylysine/DNA mixture were transgenic and no transgenic pup was born from microinjection of DNA alone into the cytoplasm. In 2003, Schmotzer *et al*. (2003) demonstated that 4.64% of zygotes were exhibited fluorescence injected with linear DNA and followed by electropulsation. However, these procedures decrease development score dramatically. Even though some researchers yet believe that this method should be more superiority compared to other transgenic methods because of it's multifarious advantages such as quick, easy, economic, convenient and huge survival rate post-injection.

The objective of this research was to demonstrate the parameters of the high efficiency method. Researchers examined three influencing factors on embryonic development and transgenic efficiency following cytoplasmic injection using pEGFP-N1 plasmid including DNA concentration, DNA volume and injection timing. During these experiments, some embryos expressing EGFP were observed under fluorescence microscope and transplanted to 11 recipients and one of them became pregnant and gave birth to two transgenic rabbits.

MATERIALS AND METHODS

Animal and zygotes harvested: The donor rabbits were induced to superovulate with six 1.2 mg sc injection of Follicle Stimulating Hormone (FSH) given 12 h apart, followed by an iv injection of 100 IU of human Chorionic Gonadotropin (hCG) given 12 h after the last FSH injection (Wright *et al*., 1981). Then, they were mated with a male rabbit and left overnight in a single cage to ensure complete fertilization. Zygotes were flushed from oviducts at 16-20 h after mating through a surgery. The zygotes in TCM199 supplemented with 10% Fetal Bovine Serum (FBS) were put at 38.5°C in a humidified atmosphere of 5% CO_2 in air for cytoplasmic injection.

DNA preparation: The transgenic cassette, a 4.7 kb pEGFP-N1 plasmid containing the *EGFP* gene drive by the CMV promoter was purified and condensed according to the description of non-endotoxin kit (QIAGEN), resuspended in ddH_2O overnight at 4°C. The purified DNA was quantified by NanoDrop 1000 spectrophotometer, diluted to 100 ng μL^{-1} in TE buffer, aliquoted and stored at -20°C until used.

Cytoplasmic injection: Zygotes were placed into the injection chamber, a line 10 μL droplet in the center of a 60 mm dish. Mineral oil was gently layered until covering the drops on the dish, decreasing loss of medium through evaporation. The EGFP construct was loaded into a microinjection needle with the inside diameter of 3-4 μm and injected into the cytoplasm. Special care was taken to ensure that the injection needle did not puncture into the pronuclear membranes of the zygotes.

***In vitro* culture, determination of EGFP fluorescence and embryo transfer:** The surviving zygotes were cultured in TCM199 supplemented with 10% FBS at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Half of the culture medium was replaced 1 day of the interval. The cleavage and blastocysts rates were observed on day 2 (cleaved) and day 7 (blastocysts). During *in vitro* culture, embryos were briefly exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm to determine the expression of the *EGFP* gene at different stages of development (Fig. 1). Furthermore, researchers selected 289 two-cell zygotes according to the morphology and respectively transplanted them into 11 estrus synchronization recipients.

Design of experiments: In order to establish an efficient procedure for producing transgenic embryos, several trials were performed. Zygotes were randomly assigned to different groups to investigate the influence factor of developmental potentiality and transgenic efficiency. Experiments 1: effect of different concentrations of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression. Experiments 2: effect of different volumes of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression. Experiments 3: effect of harvested at different times for cytoplasmic injection on embryonic development and EGFP expression.

Integration and expression analysis of *EGFP* gene in tissues: The two survival pups (No. 1 and 2) were given birth after embryo transplantation. Genome was extracted from organs (kidney, lung, heart, liver and muscle)

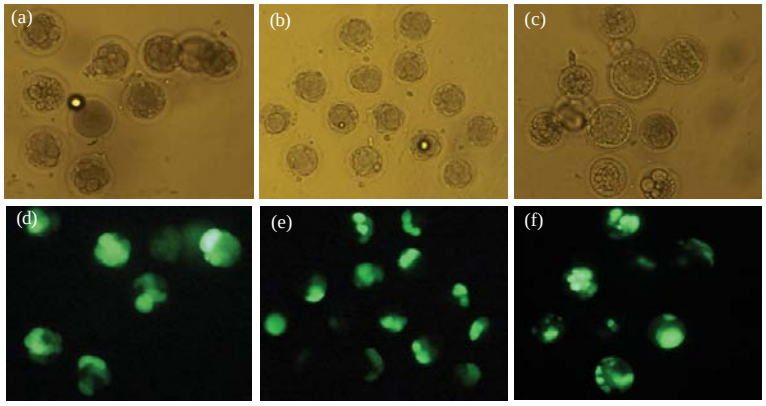

Fig. 1: Embryos were briefly exposed to blue light using an excitation filter at 488 nm and an emission filter at 530 nm at different stages of developmentat different developmental stages; a-c) bright-field pictures of developing cytoplasmic injection produced EGFP-positive embryos and d-f) corresponding pictures under fluorescent light exposure (x100)

of transgenic fetuses produced by cytoplasmic injection, performed by PCR using a pair of primers (5'-GATCATTGGAGGAGAGATTGCCAGTG-3', 5'-GTCTTGTAGTTGCCGTCGTCCTT-3'). A 720 bp production would be amplified with 2 min at 94°C, 34 cycles for 30 sec at 94°C, 30 sec at 60°C and 45 sec 72°C and finally 72°C/5 min.

Several of tissues of transgenic fetuses were sliced with freezing microtome (Leica CM3050s). The frozen tissue sections were subjected to confocal laser scanning microscope at 488 nm to observation the expression of EGFP. Furthermore, Western blot was carried out to examine the the expression of EGFP of the tissues as described by Tomomura *et al.* (2001). The proteins recovered from tissues was separated by SDS-PAGE and electrophoretically transferred onto an nylon membrane then was blocked overnight in TBST containing 5% non-fat milk at 4°C, incubated with a polyclonal anti-GFP antibody (anti-GFP antibody, 1:5,000 dilution) then washed and incubated with a secondary antibody (with HRP conjugated goat anti mouse IgG, 1:1,000), washed again and visualized by chemiluminescence detection.

RESULTS AND DISCUSSION

Effect of different concentrations of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression: Researchers evaluated four concentrations of pEGFP-N1 plasmid (30, 40, 60 and 75 ng μL^{-1}) to be injected in zygotes at one-cell stage. The result showed that the higher EGFP-positive embryos rates were obtained using concentrations of 60 ng μL^{-1} (64.6%) and 75 ng μL^{-1} (58.8%) compared to those of the groups of 30 ng μL^{-1} (0) and 45 ng μL^{-1} (10.2%) ($p<0.05$) and there was no significant difference between the former two ($p>0.05$). However, in term of embryonic development, the data showed that injection DNA with the concentration of 75 ng μL^{-1} had a significantly lower morula and blastocyst rates than that of the 60 ng μL^{-1} group (33.9 vs. 54.1%, 30.4 vs. 49.0%, $p<0.05$). In summary, considering both of transgenic efficiency and embryonic development, concentration of 60 ng μL^{-1} was the optimal concentration for cytoplasmic injection to produce transgenic embryos (Table 1).

Table 1: Effects of different concentrations of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression

Concentration (ng μL^{-1})	No. of zygotes	No. of morula (%)	No. of blastocysts (%)	No. of EGFP-positive blastocysts (%)
30	72	39 (54.2)^{a}	36 (50.0)^{a}	0 (0.0)^{c}
45	97	55 (56.7)^{a}	49 (50.5)^{a}	5 (10.2)^{b}
60	98	53 (54.1)^{a}	48 (49.0)^{a}	31 (64.6)^{a}
75	112	38 (33.9)^{b}	34 (30.4)^{b}	20 (58.8)^{a}

$^{a-c}$Means with different superscripts are significant difference in the same column ($p<0.05$)

Effect of different volumes of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression: Researchers evaluated three scale (40, 50 and 60 pL, volume was calculated using volume formulas for cylinder, $V = \pi r^2 h$, r = ID/2, h, injection length recorded using a calibration eyepiece) to determine the optimal volume for cytoplasmic injection, group injected with buffer only as control. The result demonstated that groups injected with 40 pL buffer only and 40 pL pEGFP-N1 plasmid obtained higher rates of morula (62.3 and 61.6%) and blastocysts (55.8 and 54.5%) compared with the groups of 50 pL (44.1 and 25.4%) and 60 pL (33.1 and 18.7%) ($p<0.05$). However, there were

Table 2: Effects of different volumes of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression

Volume	No. of zygotes	No. of morula (%)	No. of blastocysts (%)	No. of EGFP-positive blastocysts (%)
Buffer	77	48 (62.3)[a]	43 (55.8)[a]	0 (0.0)
40 pL	99	61 (61.6)[a]	54 (54.5)[a]	32 (59.3)
50 pL	118	52 (44.1)[b]	39 (33.1)[b]	18 (46.2)
60 pL	134	34 (25.4)[c]	25 (18.7)[c]	12 (48.0)

Table 3: Effects of harvested at different times for cytoplasmic injection on embryonic development and EGFP expression

Zygotes harvest time (h post-mating)	No. of zygotes	No. of morula (%)	No. of blastocysts (%)	No. of EGFP-positive blastocysts (%)
16	107	58 (54.2)	51 (47.7)	35 (68.6)[a]
18	115	53 (46.1)	49 (42.6)	22 (44.9)[b]
20	92	37 (40.2)	31 (33.7)	3 (9.7)[c]

[a-c]Means with different superscripts are significant difference in the same column ($p<0.05$)

no significant differences among the three treatment groups in EGFP expression at blastocyst stage (59.3, 46.2 and 48.0%) (Table 2).

Effect of harvested timing for cytoplasmic injection on embryonic development and EGFP expression: The results regarding embryonic development and transgenic efficiency after zygote cytoplasmic injection which harvested at different timing (16, 18 and 20 h) were summarized in Table 3. It showed that there was a descended tendency on rates of embryonic development and EGFP-positive embryos with the prolonged timing of zygotes recovery.

Recovering the zygotes at 16 h resulted in the highest fluorescent blastocyst rate (68.6%) which was significantly different contrasting to 18 h (44.9%) and 20 h (9.7%) groups. Embryonic development was also evaluated at morula and blastocyst stages. The outcome indicated that the series of experiments had no significant difference in developmental ability.

Detection of exogenous DNA: The integration of the pEGFP-N1 transgene in tissues from transgenic founders was analyzed by PCR. Control group was performed using tissues from wide type rabbit. The expected 720 bp specific band were observed after genomic DNA separated by agarose gel electrophoresis and with none band in wide type rabbit tissues (Fig. 2a and b). However, the result showed that both of the two transgenic rabbit were mosaic which the expression of EGFP was observed only in liver and kidney tissues.

Results of frozen tissue sections of the two founders' analyzed by confocal laser scanning microscope also showed that expression of EGFP was also observed only in kidney and liver of both transgenic founders but not in other tissues (Fig. 3). Moreover, the result was consistent with Western blot (Fig. 3b).

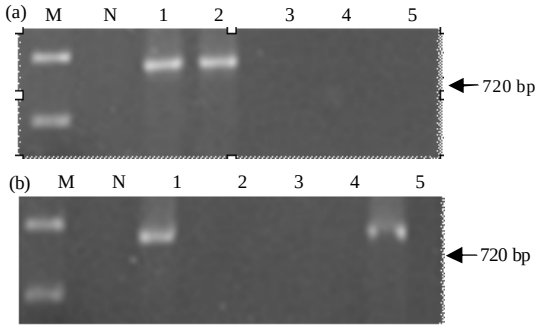

Fig. 2: The transgene was detected by PCR in two pups. The 720 bp, the PCR production of *EGFP* gene was amplified by primers (5'-GATCATTGGAGGAGAGATTGCCAGTG-3' and 5'-GTCTTGTAGTTGCCG TCGTCCTTCCTT-3'). a) No. 1, lane M to 6: Marker, negative, liver, kidney, heart, lung and muscle, b) No. 2, lane M to 6: Marker, negative, liver, lung, lung, heart and kidney

The rabbit is phylogenetically closer to primates than rodents and is large enough to permit non-lethal monitoring of physiological changes. The transgenic rabbit system fills an important niche between the laboratory mouse and larger domesticated mammals on an experimental and a commercial scale. Furthermore, the pronuclei of rabbit and mouse zygotes are visible at one-cell stage and it is bright in their cytoplasm without lipids which can avoid hurt when cytoplasmic injection. However, the mouse zygotes are more sensitive to mechanical manipulation during injection than rabbit zygotes. Comparison of rabbit and mouse zygotes revealed differences in the survival rate after microinjection. A higher percentage of rabbit zygotes (85.4%) survived than that of mouse (43.2%) during the mechanical manipulation of the microinjection procedure (Voss *et al*., 1990). So, rabbits are optimal model animal for transgenic research using method of cytoplasmic injection. In the present study, researchers evaluated the effects of related factors with cytoplasmic injection such as concentration of plasmid, volume for injection and timing for zygotes harvesting.

The data obtained from zygotes injected with different of plasmid concentrationas showed that 60 ng μL^{-1} was the optimal concentration for injection with which approximately half of the zygotes develop to blastocyst and 64.6% blastocyst expressing EGFP. While the concentration of 75 ng μL^{-1} was performed, researchers obtained extraordinary low blastocysts rate compared to the group injected with lower concentration of DNA despiting the high transgenic efficiency.

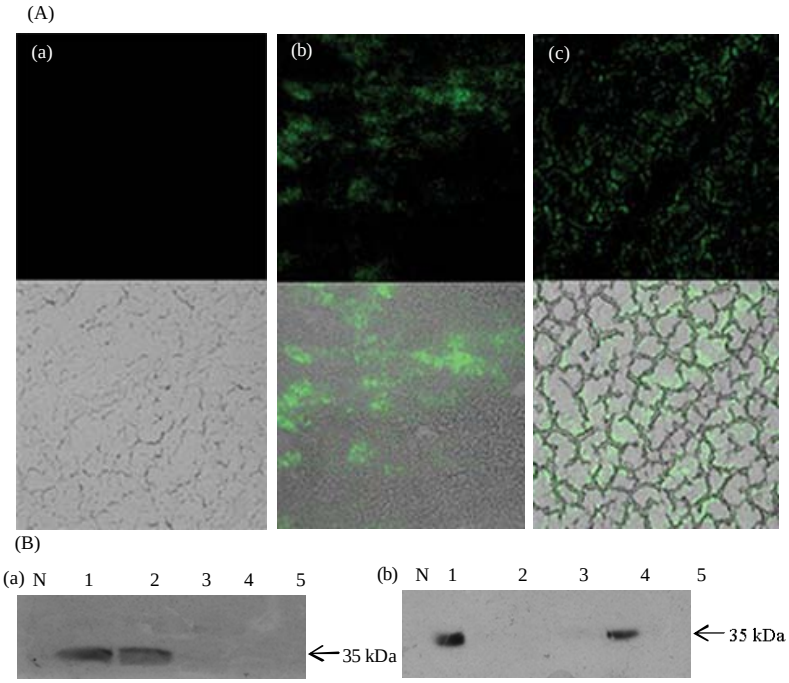

Fig. 3: A) EGFP expression of frozen sections were detected by confocal laser scanning microscope under bright-field and fluorescent light, a) negative, b) kidney and c) liver. B) Transgenic detection by Western blot, a) tissues dissected from No. 1, N to 5, negative, liver, kidney, heart, lung, muscle; b) tissues dissected from No. 2, N to 5 negative, liver, lung, heart, kidney, muscle. EGFP were expression only in kidney and liver both No. 1 and No. 2

Correspondently, Wu and Wu (1987) had point out that high concentration may inhibit embryonic development in pronuclear injection but it does not inhibit DNA transcription, mRNA translation and protein expression. The result indicated that the lethality of DNA is concentration dependent, the higher concentration of DNA, the lower the surviving rate of rabbit zygotes. Vichera *et al*. (2010) had proved that high concentration of exogenous DNA was degraded in the cytoplasm after cytoplasmic injection and liposomes can protect it from endonucleases until its transcription. So in the research, the reason for high concentration of exogenous DNA resulting in a reduced ability of embryonic development is that the endonucleases may be activated by excessive exogenous DNA which can degrade the embryonic genome. With regard to exogenous DNA concentration on transgenic efficiency in pronuclear injection, Page *et al*. (1995a, b) had pointed out that integration frequency was improved as concentration of exogenous DNA increases, moreover, they fond that exogenous DNA had a adverse on development of embryos *in vitro* and the adverse effect was also concentration dependent.

In the second experiments, researchers evaluated the effect of different volumes of exogenous DNA for cytoplasmic injection on embryonic development and EGFP expression. The results showed that a significant higher blastocyst rate (54.5%) and EGFP-positive blastocyst (59.3%) was obtained in the 40 pL group but the blastocyst rate was dropped dramatically with the increase of injection volume. These results indicated that injecting with 40 pL DNA are tolerable for cytoplasmic injection which was two or three folds suggested by earlier investigation with 10-15 pL. The injection of exogenous DNA into cytoplasm, irrespective of construct could cause a significant decrease in developmental potential (Page *et al*., 1995a, b). In the studies, researchers found that embryonic development is getting worse with the volume increase. These results did not agree with earlier report. Brinster *et al*. (1985) had suggested that in pronuclear injection with buffer only had no adverse effects on subsequent embryonic development to blastocyst and they led to the conclusion of not the injection event itself causing decrease in embryonic development. By comparing these results, researchers consider that distinction of between the two methods may causing a large enough amount which only injected with 1-2 pL in pronuclear injection but 10 folds increasing in cytoplasmic injection. It was thought that injecting excessive influx of exogenous substances into cytoplasm leads to decreasing in embryonic development. According to development stage of rabbit zygotes cultured *in vivo*,

zygotes still did not cleavage by the time of 14-20 h post-mating (Maurer, 1978). In general, zygotes recovered at 15-16 h post-mating had fertilized and presented a fuzzy nuclear area. In the current study, the zygotes harvested at different time (16, 18 and 20 h) after injected with exogenous DNA and the group of 16 h were acquired the highest rates of blastocysts and EGFP expression at blastocysts stage. This result indicated that the optimal injection timing should be taken before the mitosis of fertilized eggs. Iqbal *et al.* (2009) had tried to injecte cDNA encoding EGFP in the cytoplasm of murine zygotes and pointed out transcriptional activity was begin during S/G2 phase of the first cell cycle. In addition, several of earlier studies also shown that the exogenous gene expression prior to the joining of the male and female pronuclear, furthermore the male pronuclear exhibits great transcriptional activity (Adenot *et al.*, 1997). Two pups were given birth after embryo transfer.

The transgene integration and expression were analyzed by PCR, frozen tissue sections analyzed by confocal laser scanning microscope and Western blot. It is likely to cause transgene integration in a mosaic manner of EGFP. However, it is known that not only method of cytoplasmic injection but all the four basic technique can occur mosaic in transgenic animals generated. In particular, Schmotzer *et al.* (2003) reported that quite high 71% of transgenic murine embryos by pronuclear injected were mosaicism (Thrasher and Kilburn, 2001).

The pattern of transgene integration may be a random event by the fracture and enzymatic repair mechanisms of the chromosome like pronuclear injected (Brinster *et al.*, 1985). It often occurs following the first cell division (Chan *et al.*, 1999). The one of two blastomeres making the pup and the rest go to making the placenta. Niu *et al.* (2010) point out the reason of formation hight rate mosaicism was cells deriving from the infected blastomere underwent negative selection during inner-cell-mass and epiblast development. But, the result implied that it could decrease the frequencies of mosaicism through shorting the timing of harvested (12-14 h post-mating).

CONCLUSION

In summary, this rising methodology potentially offers several advantages over others technique in transgenic animals producing. Researchers believe that it will be a reliable tool for studying ectopic gene expression in transgenic animals and reprogramming events during early ontogenesis in future.

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