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Preliminary Study on Transgenesis by Injecting Exogenous DNA into Zygote Cytoplasm of Buffalo

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Abstract [**Objective**] This study aimed to investigate the feasibility of transgenesis by injecting exogenous DNA into zygote cytoplasm of Buffalo. [**Method**] Buffalo oocytes were randomly divided into two groups 20-22 h after *in vitro* maturation. One group of oocytes was introduced with about 7.5 pl of 50 μg/ml DNA solution containing linear EGFP fragment by cytoplasmic injection 7-10 h or 18-20 h after *in vitro* fertilization (IVF); the other group of oocytes was introduced with mixture of a single buffalo sperm and about 7.5 pl of 50 μg/ml DNA solution containing linear EGFP fragment by cytoplasmic injection (generally called ICSI-Mediated Gene Transfer, ICSI-Tr). Expression of exogenous DNA was observed and recorded during the process of embryonic development. [**Result**] Early embryonic gene expression efficiency and blastocyst gene expression efficiency in IVF injection group showed no significant difference compared with that in ICSI-Tr group (P>0.05). In addition, the cleavage rate and early embryonic gene expression efficiency in IVF injection group were significantly higher with injection at 7-10 h post IVF than that at 18-20 h post IVF (P<0.05). [**Conclusion**] These results indicate that transgenic buffalo embryos can be generated by injecting exogenous DNA into cytoplasm of IVF oocytes, and the optimal injection time is 7-10 h post IVF.
Key words Buffalo; Zygote; Cytoplasmic injection; Transgenesis

ICSI-mediated mammalian transgenesis is a simple and highly efficient transgenesis method, which has been successfully used in mice[1-3], rats[4] and pigs[5]. ICSI technology can also be used in the production of transgenic buffalo embryos[6]. Dead sperms killed by repeated freeze-thawing were used for ICSI-mediated buffalo transgenesis. DNA can adhere to the dead sperms but can not be internalized into the sperm nuclei[7]. During the process of ICSI-mediated transgenesis, exogenous gene may adhere to the sperms and enter cytoplasm; in addition, exogenous DNA can be integrated into zygote genome with a relatively high efficiency during the process of sperm depolymerization, pronuclear formation and prokaryotic fusion, thus achieving transgenesis. On the other hand, since the exogenous gene enters cytoplasm by adhering to the sperms, it also has the chance to contact with the chromosome. During the replication of zygote chromosomes, exogenous DNA also has an opportunity to be integrated into zygote genome, thereby achieving transgenesis. Injecting exogenous DNA into the zygote before the formation of male and female pronuclei also has the opportunity to achieve transgenesis. Buffalo oocyte cytoplasm is dark with a lot of lipid droplets, and no clear pronucleus can be observed under the microscope. Furthermore, nucleus observation by physical and chemical treatments will cause damages to oocyte cytoskeleton. Therefore, the nuclear injection method has certain limitations. No nucleus factor has to be considered in zygote cytoplasmic injection, so achieving transgenesis by injecting exogenous DNA into zygote will make the transgenic technology easier. The feasibility of producing transgenic embryos by directly injecting exogenous DNA into zygote cytoplasm of Buffalo was investigated in this study.

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Materials and Methods

Reagents

TCM199 was purchased from Gibco Corporation; other reagents were purchased from Sigma Corporation. Water used for preparing various types of culture solution was first filtered by Millipore and then distilled once with quartz sub-boiling high pure water distiller.

DNA preparation

Vectors pEGFP-N1 (Fig.1) and pPGK1-FAT1-CMV-EGFP (Fig.2) containing reporter gene CMV/EGFP were used in this study. QIAGEN Plasmid Maxi Kit was used for plasmid purification. After restriction enzyme digestion and PCR identification, the purified plasmid was digested with ApaII at 37 ℃ for 3-4 h and detected by agarose gel electrophoresis, then purified with alcohol precipitation method, dissolved by using ultrapure water, determined

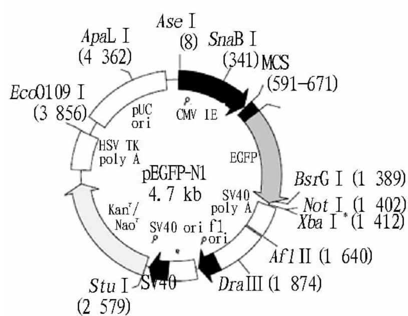

Fig.1 The plasmid pEGFP-N1

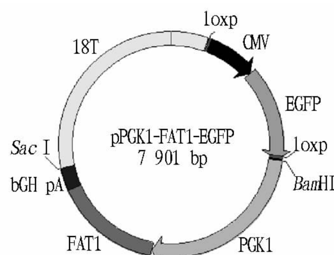

Fig.2 The plasmid pPGK1-FAT1-CMV-EGFP

with NANODROP 1000 spectrophotometer, diluted to 100 μg/ml and dispensed into micro-centrifuge tubes, 10 μl per micro-centrifuge tube, which was then preserved at –20 ℃ for use.

Sperm preparation

High-activity buffalo sperms were collected by using the swim-up method. Buffalo frozen semen (Improvement Station of Livestock Breed in Guangxi Area) was placed at the bottom of a 10 ml sterile test tube after conventional thawing; 2 ml of fertilization medium (modified Tyrode's medium without lactate, containing 50 μg/ml of heparin, 2.5 mmol/L of caffeine, 60 mg/L of penicillin, 100 mg/L of streptomycin) was collected and pre-heated in 5.0 % CO_2 incubator at 37.0 ℃ for 30 min, then added slowly into the test tube; subsequently, the test tube was placed in the incubator for sperms to swim up. After 20 min of swimming up, 1 ml of supernatant was transferred to another test tube with a pipette, added with 5 ml of fertilization medium and centrifuged at 500 g twice for *in vitro* fertilization.

Freezing and thawing: 0.1 ml of processed semen was added into 0.2 ml sterile EP tube, placed in liquid nitrogen at –196 ℃ for 3 min, and then naturally thawed at room temperature for of ICSI-Tr.

***In vitro* maturation of oocytes**

Buffalo ovary was purchased from Slaughterhouse of Nanning City to collect the buffalo cumulus-oocyte complexes. The obtained buffalo cumulus-oocyte complexes were placed into maturation medium [containing TCM-199, 5.0 mmol/L of Hepes, 26.2 mmol/L of $NaHCO_3$, 5% of OCS, 0.1 μg/ml of follicle-stimulating hormone (FSH), 60 mg/L of penicillin, 100 mg/L of streptomycin] and cultured at 38.5 ℃ under 5% CO_2 humidity conditions for 22–24 h.

***In vitro* fertilization and microinjection**

After *in vitro* maturation culture, buffalo cumulus-oocyte complexes with good growth morphology of cumulus were selected for *in vitro* fertilization, and the density was adjusted to 1.0×10^6 sperms/ml. After *in vitro* fertilization for 7–10 or 18–20 h, second polar body-extruded zygote with uniform cytoplasm was selected, and then about 7.5 pl of DNA solution was injected into the zygote cytoplasm with ICSI injection needle (diameter of about 9–10 μm). After injection, the zygote was washed twice with 2 ml of embryo medium (Culture Medium, CM; containing TCM–199, 5.0 mmol/L Hepes, 26.2 mmol/L $NaHCO_3$, 3% of OCS, 60 mg/L penicillin, 100 mg/L of streptomycin), and then cultured in the incubator.

ICSI-Tr

After *in vitro* maturation, buffalo cumulus-oocyte complexes were transferred into CM containing 0.1% of hyaluronidase and beaten upon gently with a pipette to remove the cumulus cells, and then first polar body-extruded mature oocytes with uniform cytoplasm were selected for ICSI-Tr (ICSI-Mediated Gene Transfer).

Single sperm injection needle diameter is 9–10 μm and fixed needle diameter is 20 μm, which were both purchased from the HUMAGEN Company (USA). A tube of frozen and dispensed pEGFP-N1 plasmid was recovered at room temperature before ICSI, mixed with equal volume of sperm suspension for 5 min and added with 7% PVP solution (SAGE, USA); subsequently, the mixture of sperm and DNA was injected into MII phase oocytes of buffalo in accordance with the method proposed by Zhang *et al.* [8] In addition, some buffalo mature oocytes were only injected with exogenous DNA solution but not sperms (sham injection). After injection, ICSI oocytes were washed twice with CM solution and placed in the incubator for activation.

Chemical activation of oocytes

After ICSI and sham injection, the oocytes were cultured for 1h in the incubator for activation. The oocytes were first activated with CM containing 5 μmol/L ionomycin (ION) for 5 min, then cultured with CM containing 10 μg/ml cycloheximide (CHX) for 5 h, washed twice with 1 ml of CM, and transferred into a petri dish for culture.

Embryo culture

Activated oocytes, sham-injected oocytes and IVF oocytes were co-cultured with cattle granulosa cells at 38.5 ℃ under 5% CO_2 humidity conditions in the incubator. CM solution was replaced every 48 h. The cleavage rate was determined after 24 h and the blastocyst development rate was recorded after 7 d.

Examination of transgenic positive embryos

Embryos were placed under a fluorescence microscope to detect the expression of *EGFP* in embryos. The number of embryos express *EGFP* at different developmental stages was recorded.

Experimental design

Experiment I was conducted to explore the feasibility of expressing exogenous gene by injecting exogenous DNA into IVF zygotes of buffalo. About 7.5 pl of 50 μg/ml pEGFP-N1 plasmid was injected into second polar body-extruded buffalo oocytes at 7–10 h post IVF, to observe the expression of *EGFP* during the process of embryonic development and compare with ICSI-Tr and sham-injected oocytes.

Experiment II was conducted to explore the effect of injection time post IVF on expression of exogenous gene. About 7.5 pl of 50 μg/ml Fat-1 plasmid was injected into second polar body-extruded buffalo oocytes respectively at 7–10 h post IVF and 18–20 h post IVF, to observe the expression of Fat-1 during the process of embryonic development.

Statistical analysis

All the experimental data were analyzed with chi-square test (x^2) to determine the significance of the difference.

Results and Analysis

Transgenesis by injecting exogenous DNA into buffalo IVF zygotes

As shown in Table 1, the cleavage rate of IVF injection group was significantly lower than that of ICSI-Tr group and sham injection group (P<0.01); the blastocyst development rate of IVF injection group had no significant difference from that of ICSI-Tr group (P>0.05), while the blastocyst development rates of ICSI-Tr group and sham injection group were significantly different (P <0.01). The early embryonic gene expression efficiency of IVF injection group had no significant difference from that of ICSI-Tr group (P>0.05). The early embryonic gene expression efficiency of sham injection group was significantly lower than that of IVF injection group and ICSI-Tr group (P<0.01). These results suggest that exogenous gene can be expressed in IVF embryos by injecting exogenous DNA into buffalo IVF zygotes, and the expression efficiency is consistent to the ICSI-Tr group; injecting exogenous DNA into MII phase oocytes of buffalo can also achieve the expression of exogenous gene in parthenogenetic embryos, but the early embryonic gene expression efficiency is significantly lower than that of ICSI-Tr and IVF injection.

Effects of IVF time on exogenous DNA expression by zygote cytoplasmic injection

As can be seen from Table 2, the cleavage rate and early embryonic gene expression efficiency at 18–20 h post IVF were both significantly lower than 7–10 h post IVF (P<0.01; P<0.05); the blastocyst development rate and blastocyst gene expression efficiency were both reduced, with no significant difference. Therefore, exogenous DNA injection at 7–10 h post IVF can achieve better transgenesis result than exogenous DNA injection at 18–20 h post IVF.

Conclusion and Discussion

The results show that transgenic embryos can be obtained by injecting exogenous genes into IVF zygote cytoplasm of buffalo, and the effect of exogenous DNA injection at 7–10 h post IVF is better than that at 18–20 h post IVF.

In 1980, Gordon *et al.*[9] first reported obtaining transgenic mice by DNA microinjection. Due to the stable results and good repeatability, pronuclear microinjection is still the most widely used and the most mature transgenic method. Mouse and rabbit ooplasm contains few lipid, the egg is relatively transparent, and the pronucleus can be observed clearly, which is easy for pronuclear microinjection to achieve high success rate of transgenesis. However, ooplasm of cattle, sheep, buffalo, pig and other livestock contains more lipid droplets, the ooplasm is relatively dark, and the pronucleus can not be observed clearly, which is difficult for pronuclear microinjection, with low transgenesis efficiency. In 1999, Perry *et al.*[1] reported the program that using ICSI technology to obtain transgenic mammalian with sperms as the carrier of exogenous gene based on the characteristics of pronuclear microinjection and sperm carrier and successfully obtained transgenic mice. ICSI-mediated transgenic strategy not only reduces the technical difficulty, but also has the transfection efficiency no less or even higher than pronuclear microinjection, which provides new ideas for livestock transgenesis and has been successfully applied in pig transgenesis.

ICSI technology for large livestock such as cattle, sheep and buffalo is immature, with difficult sperm depolymerization, low fertilization rate and success rate, which will restrict the application of ICSI-mediated transgenic technology. However, IVF technology for large livestock is relatively mature, achieving transgenesis by injecting exogenous DNA into zygotes will further simplify the procedures of transgenic technology and improve the transgenic efficiency of large livestock.

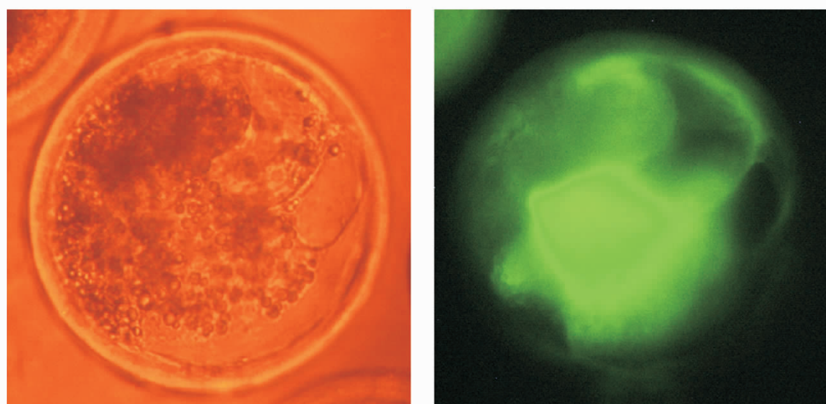

Fig.3 *EGFP*-expressing blastocyst obtained by injecting pEGFP-N1 plasmid into buffalo zygotes (200 ×)

Table 1 Development and gene expression of buffalo IVF embryos injected with exogenous DNA

Groups	Number of oocytes	Cleavage rate // %	Early embryonic gene expression efficiency // %	Blastocyst development rate // %	Blastocyst gene expression efficiency // %
IVF injection	116	69.0 a	48.8 a	15.5	50.0
ICSI-Tr	122	89.3 b	41.3 a	20.5 a	56.0
Sham injection	108	87.0 b	14.8 b	7.4 b	100.0

Different letters indicate significant difference. a, b: P<0.01

Table 2 Effects of IVF time on the transgenesis by injecting exogenous DNA into buffalo zygote cytoplasm

IVF time // h	Number of oocytes	Cleavage rate // %	Early embryonic gene expression efficiency // %	blastocyst development rate // %	Blastocyst gene expression efficiency // %
7–10	136	75.0 a	49.0 a	15.4	42.9
18–20	73	57.5 b	26.2 c	9.6	14.3

Different letters indicate significant difference. a, b: P<0.01; a, c: P<0.05

Page *et al.*[10] reported that injecting merely exogenous DNA into zygote cytoplasm can not obtain transgenic mice, but injecting the mixture of exogenous DNA and polylysine can obtain transgenic mice with the same efficiency as pronuclear microinjection. Zhang *et al.*[11] inserted the target gene expression component of bovine α-sl-casein-hG-CSF and the mammalian nuclear localization gene expression component into zygote cytoplasm by using the cytoplasmic coinjection method and obtained transgenic mice by introducing the target gene expression component into pronucleus using the directional migration function of nuclear localization protein. This transgenic approach can not only improve the birth rate of transgenic mice, but also reaches the integration rate of 58% (29/50) and the expression rate of 75%, whey at expression levels of 60–550 mg/L can be preliminarily estimated with the Western blotting method. Therefore, coinjection with exogenous DNA and polylysine and coinjection with exogenous DNA and mammalian nuclear localization gene expression component can both successfully obtain transgenic mice.

In this study, Experiment I shows that exogenous DNA injected into buffalo IVF zygote cytoplasm can be expressed in early embryos (Fig.3), and the expression efficiency is consistent to ICSI-mediated transgenesis (P < 0.05), which suggests that merely injecting exogenous DNA into buffalo IVF zygote cytoplasm can at least determine the exogenous gene expression in early embryos, while whether the expression can be integrated into future generations requires further study. In addition, Experiment I also shows that exogenous DNA injected into buffalo MII phase oocytes can be expressed in parthenogenetic embryos, which is inconsistent with the researches of mice ICSI-Tr conducted by Perry *et al.*[1] and Yamauchi *et al.* and the researches of sheep, pig, cat, cattle and horse ICSI-Tr conducted by Pereyra-Bonnet *et al*[12]. Perry believes that the EGFP-expressing embryos can be obtained only by coinjection with exogenous DNA and sperms. Result of Experiment I in this study might be due to the relatively high amount of injected exogenous DNA and the appropriate injection timing, therefore the exogenous DNA obtains the opportunity to contact with the egg genome and sperm genome before the formation of male and female pronuclei and achieves integration during DNA replication.

After capacitation, the sperm enters the egg through corona radiata, zona pellucida and vitelline membrane. Shortly, the head begins to expand, the nuclear become loose, and the nuclear membrane disappears, losing the natural form. In addition, the oocyte restores meiosis and extrude the second polar body; furthermore, the nuclear basic proteins and the high concentrations of arginine closely related to sperm DNA completely disappeares, the nuclear membrane forms outside the loose chromatin finally, with a somatic cell nucleus-like structure rather than sperm nucleus, and this re-formed nucleus is called the male pronucleus[13]. Lu[14] reported that buffalo sperm entered the eggs at 4–6 h post IVF, generated male pronucleus at 10 h post IVF, while the male and female pronuclei fused at 14–18 h post IVF. In this study, Experiment Ⅱ shows that the cleavage rate and early embryonic gene expression efficiency of exogenous DNA injection at 18–20 h post IVF are both significantly lower than that at 8–10 h post IVF (P<0.01; P<0.05), suggesting that the injection timing has great effect on transgenesis, the sperm might has not entered the egg during too early injection, while the pronucleus might has formed during too late injection, decreasing the opportunity for exogenous DNA to contact with the genomes or leading to pronucleus damages in injection, thus reducing the developmental capacity.

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敖汉细毛羊Ⅰ型内根鞘角蛋白基因表达模式的研究分析

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摘 要 [目的]探讨细毛羊毛囊生长期及休止期特异基因的表达模式以及该模式对敖汉细毛羊选育的意义。[方法]通过基因芯片技术对敖汉细毛羊羊毛生长期和休止期的颈部、腹股沟部的上皮基因表达进行检测。[结果]统计分析Ⅰ型内根鞘角蛋白基因 *KRT*25、*KRT*26、*KRT*27 和 *KRT*28 在细毛羊颈部与腹股沟部的表达,发现该基因在细毛羊生长期颈部表达量极显著高于腹股沟部的表达量(差异倍数>2,P<0.01);对比分析生长期与休止期该基因的表达变化，结果显示在腹股沟部所有芯片涵盖的Ⅰ型内根鞘角蛋白基因的表达都表现出由生长期进入休止期的显著下调（P<0.05),除 *KRT*25(*LOC*443079)外,其他基因的表达变化都在 2 倍以上。[结论]该研究结果表明Ⅰ型内根鞘角蛋白基因家族的表达与特异部位羊毛密度的控制以及整个毛囊发育生长周期都有着重要的联系。

关键词 Ⅰ型内根鞘角蛋白;内根鞘;毛纤维;细毛羊;差异表达

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水牛受精卵胞质内注射转基因的初步研究

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摘 要 [目的]旨在探讨通过向水牛受精卵胞质内注射外源 DNA 实现转基因的可行性。[方法]水牛卵母细胞体外成熟 20~22 h 后随机分为 2 组，①在体外受精 7~10 或 18~20 h 后向卵胞质内注入约 7.5 pL 50 μg/ml 含线性 EGFP 片段的 DNA 溶液；②则分别注入单个精子与约 7.5 pL 50 μg/ml 含线性 EGFP 片段的 DNA 混合物，观察外源基因在胚胎发育过程中的表达情况。[结果]受精卵胞质内注射的早期胚胎基因表达率、囊胚基因表达率与 ICSI–Tr 差异不显著(P>0.05),且 IVF 7~10 h 时注射的分裂率、早期胚胎基因表达率均显著高于 18~20 h（P<0.05）。[结论]水牛 IVF 受精卵胞质内注射外源基因能获得转基因胚胎,且 IVF 后 7~10 h 注射的效果优于 IVF 后 18~20 h 注射。

关键词 水牛;受精卵;胞质内注射;转基因

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About Agricultural Science & Technology

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According to statistics, the immediacy index of the journal has reached 3.302 in 2010. More than 80% of papers are fund papers, and most of them are supported by projects above the provincial level.To further improve the quality and impact of the publication, the journal editorial staff visited the Institute for Scientific Information (ISI) in October 2008, and they met with a senior editor Ms. Maureen Handel and her supervisor Ms. Mariana Boletta, who are responsible for the review of agricultural scientific journals included in Science Citation Index (SCI). The two sides communicated for the selection criteria of SCI included scientific and technical journals as well as the editing and publishing related matters of Agricultural Science & Technology.