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Production of Transgenic Goats by Sperm-mediated Exogenous DNA Transfer Method*

Yongju Zhao[1], Hong Wei[1,] **, Yong Wang[1], Lingbin Wang, Mingju Yu, Jingsheng Fan, Shuangyan Zheng and Cong Zhao

College of Animal Science and Technology, Chongqing Key Laboratory of Forage & Herbivore, Southwest University, Beibei, Chongqing 400716, China

ABSTRACT : In this study, the production of transgenic goats using sperm to integrate exogenous DNA and artificial insemination (AI) was carried out and the technical protocols for sperm-mediated gene transfer (SMGT) in the goat were optimized. The standard sperm parameters and the ability to bind foreign genes were assessed to select suitable sperm donor bucks. A total of 134 oestrous does were divided into 4 groups and inseminated using different methods and sperm numbers. The does of Groups I to III were inseminated with fresh semen ($1\sim2\times10^7$ and 10^6 sperm) or frozen-thawed semen (10^6 sperm), respectively, through conventional intra-cervical AI, and the does of Group IV with frozen-thawed semen (10^6 sperm) through intrauterine AI. Total genomic DNAs were extracted from ear biopsies of the offspring. The presence of pEGFP-N_1 DNA was screened by PCR and then by Southern blotting analysis. A total of 76 live kids were produced and 8 kids were tested transgene positive on the basis of agarose gel electrophoresis of the PCR-amplified fragment. Southern blotting analysis of the samples showed 5 positive kids. A transgenic ratio of 10.53% was detected using PCR and 6.58% using Southern blotting. The positive kid rate assayed by PCR and Southern blotting of frozen-thawed goat semen was 3.61% and 9.27% higher than that of untreated semen. The results show that transgenic goats can be produced efficiently by the method of artificial insemination using sperm cells to integrate the exogenous DNA and intrauterine insemination allowed low numbers of DNA-transfected spermatozoa to be used, with satisfactory fertility. (**Key Words :** Sperm-mediated Gene Transfer, Goat, Sperm Cryopreservation, Artificial Insemination (AI))

INTRODUCTION

Transgenic technology holds considerable promise to advance understanding in biomedical and agricultural systems with some believing that one day transgenic animals may directly contribute to farming and breeding practice. Practical applications of transgenics in livestock production include improved milk production and composition, increased growth rate and disease resistance, improved feed usage and carcass composition, enhanced reproductive performance and increased prolificacy (Houdebine, 2002; Bacci, 2007; Venkatesh, 2008; Zdunczyk and Pareek, 2009). Recently, transgenic technology has also provided a method to transfer nutritionally beneficial traits to other foodstuffs (Arihara, 2006; Maga et al., 2006). The first transgenic livestock were born 20 years ago (Hammer et al., 1985) and there are numerous potential transgenic methodologies to generate transgenic animals. Today, the most widely used methods for the production of transgenic farm animals are direct microinjection of foreign DNA into the pronuclei of fertilized eggs, nuclear transfer using genetically modified embryonic or somatic donor cells and viral-based constructs as vectors for the introduction of exogenous DNA into embryos. These methods have been restricted in part by inefficiency when applied in livestock. Moreover, it must be noted that the use of retroviral vectors is affected by safety issues (Lavitrano et al., 2006; Whitelaw et al., 2008).

The spontaneous capability of sperm cells to bind exogenous DNA molecules and internalize them into nuclei can be exploited by using spermatozoa as vectors for

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** Corresponding Author: Hong Wei. Tel: +86-23-6875-2051, Fax: +86-23-6875-2051, E-mail: y.zhaoswu@163.com

[1] Faculty of Laboratory Animal, The Third Military Medicine University, Shapingba, Chongqing 400038, China.

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delivering foreign genetic information to eggs during fertilization (Brackett et al., 1971; Maione et al., 1998). Sperm-mediated gene transfer (SMGT) appears to be a simple, efficient and relatively inexpensive method for modifying animals and their genome. It is also potentially applicable to every species using spermatozoa for its own propagation. Lavitrano et al. first described the SMGT procedure in mouse with high efficiency. Subsequently, they successfully adapted and optimized the technique for use in large animals. It was highly efficient for the generation of human decay accelerating factor (hDAF) transgenic pig lines (Lavitrano et al., 1989; Lazzereschi et al., 2000; Lavitrano et al., 2002). Several studies have proven the broad applicability of this technique to different animal species, from sea urchin to cattle (Lavitrano et al., 1989; Camaioni et al., 1992; Francolini et al., 1993; Nakanishi and Iritani, 1993; Lavitra et al., 1997; Maione et al., 1998; Khoo, 2000; Smith and Spadafora, 2005; Hoelker et al., 2007; Moisyadi et al., 2009). DNA uptake by mammalian spermatozoa is a very specific and highly regulated phenomenon. A considerable number of studies performed since then have revealed some unexpected aspects of spermatozoa, which suggest that such an attractive route to transgenesis may not be so easy but could be still be available if only the underlying mechanisms were understood (Brinster et al., 1989; Gandolfi, 1998; Spadafora, 1998).

Because of the outstanding protein synthetic capacity of the mammary gland and appropriate body size, the goat (*Capra hircus*) is the ideal animal for transgenics to produce recombinant proteins in a more efficient manner than other livestock. However, at present there are few reports of transgenic goats by SMGT. This study was carried out in order to establish technical protocols for SMGT for goats and evaluate the possibility of producing transgenic goats by the method of artificial insemination (AI) using sperm cells to integrate the exogenous DNA.

MATERIALS AND METHODS

Experimental animals and management

This study was conducted at Rongchang goat farm (in western Chongqing City), Xichong goat farm (in northern Sichuan province). The bucks (sperm donors) were housed at the facilities of The Third Military Medicine University. The bucks were Boer, Yudong White goat and Nanjiang Yellow goat. The doe breeds were Guanzhong Dairy goat and Nanjiang Yellow goat. The does that had kidded at least once, aged between 2 and 6 years and weighing 28-40 kg, were group-housed under natural photoperiod. The does were allowed access to an outdoor concrete run and fed a daily ration of 200-300 g concentrate. In addition, all experimental goats were allowed to an unrestricted access to straw, mineral salt lick and water.

Experimental design

In order to set up and compare the technique with conventional processed semen, and to evaluate the effect of the reduction of spermatozoa number on fertilization rate, a total of 134 estrus induced does were divided into 4 groups randomly (Table 1).

Plasmid preparation and DIG DNA labeling

The plasmid pEGFP-N1 encoding green fluorescent protein reporter gene was prepared at a large scale as described by Sambrook and Russell (2001), and extracted by plasmid maxi kit (Omega Biotek, Norcross, GA, USA). The extracted plasmid was digested by ApaII or NotI (Roche, Basel, Switzerland), and resuspended in filtered sterilized water.

Some plasmid DNA was digested by Hind III (Roche) and Dig Labeled. The DIG DNA Labeling was according to the kit protocol (DIG DNA Labeling and Detection Kit, Roche). Briefly, lineared plasmid DNA was diluted in autoclaved, double distilled water to a total volume of 15 μl and denatured by heating for 10 min in a boiling water bath and quickly chilled on ice before adding 2 μl hexanucleotide mix, 2 μl dNTP mixture and 1 μl Klenow enzyme. This was mixed and centrifuged briefly, then incubated at 37°C overnight. The reaction was stopped by adding 2 μl EDTA (0.2 mol/L, pH 8.0). The labeling efficiency was quantified with a dot blotting method on one strip of nylon membrane. A dilution series of the labeled DNA and the control DNA were prepared as described in the kit.

Semen collection and processing

Semen samples were collected with a pre-warmed artificial vagina. The treatment and washing procedure was as described previously (Zhao et al., 2005). The ejaculates

Table 1. The number of experimental does, difference in insemination deposition site and sperm number

Group	Number of does	Artificial insemination deposition site	Fresh/frozen	Number of motile spermatozoa
I	48	Intra-cervical	Fresh	$1\text{-}2\times10^7$
II	29	Intra-cervical	Fresh	10^6
III	16	Intra-cervical	Frozen-thawed	10^6
IV	41	Intrauterine	Frozen-thawed	10^6

were immediately diluted 1:4(v/v) in pre-warmed TALP buffer and washed (500 *g* for 5 min) at room temperature. Then, one aliquot of each ejaculate was immediately transported to the laboratory. Semen cryopreservation was performed as described (Zhao et al., 2008). For cryopreservation, briefly, the spermatozoa were immediately extended with Tris buffer (Tris 4.039%, citric acid 2.316%, glucose 0.667%, egg yolk 20%, glycerol 5.3%, penicillin 100,000 IU/100 ml, streptomycin 100,000 IU/100 ml) at a concentration of 200×10^6 spermatozoa. After that, the samples were maintained at 2-5°C for 4 h. Finally, the aliquot was cryopreserved as 0.1 ml pellets and stored in liquid nitrogen (-196°C) for at least 15 days. The pellets were thawed in TALP buffer at 40°C for 1-2 min.

Incubation of goat sperm with DNA *in vitro* and assessment of DNA uptake

Goat sperm were incubated with DNA *in vitro* as described in (Zhao et al., 2005). In short, semen (fresh or frozen-thawed) was immediately diluted in pre-warmed TALP buffer and washed three times at room temperature. About 10^6 sperm cells were incubated with 1,000 ng plasmid DNA at 20°C in TALP buffer for 60 min.

The DNA-treated sperm were washed three times in 0.9% NaCl and then fixed in 4% paraformaldehyde in PBS for 30 min. The sperm were smeared on gelatin-coated slides, dried and fixed. The sperm slides were rinsed in 0.01 mol/L phosphate-buffered saline (PBS) twice then in Washing buffer (Tris-HCl 100 mmol/L, NaCl 150 mmol/L, pH 7.5) for 10 min. They were then incubated in Blocking solution (Dissolve Blocking reagent 10% (w/v) in washing buffer while constantly stirring on a heating block (65°C) or heat in a microwave oven, then autoclave. Prepare a 1×working solution by diluting 10×Blocking solution 1:10 with Washing buffer) for 30 min. Anti-Digoxigenin-AP was prepared by centrifuging for 5 min at 10,000 *g*, then pipetting the necessary amount carefully from the surface and diluting it 1:1,000 in Blocking solution. Binding to the DIG-labeled probe in Antibody solution was performed for 30 min, followed by equilibration for 5 min in Detection buffer (Tris-HCl 100 mmol/L, NaCl 100 mmol/L, pH 9.5) and incubation in 2 ml freshly prepared Colorsubstrate solution (Add 200 µl of NBT/BCIP to 10 ml of Detection buffer) for 60 min in the dark. The reaction was stopped in TE-buffer (Tris-HCl 10 mmol/L, EDTA 1 mmol/L, pH 8.0) and the slides washed for 5 min with 50 ml double distilled water. Finally, slides were examined and photographed under a microscope (Olympus CX41) at 1,000× magnification.

Synchronized estrus induction

The does were treated using intravaginal sponges impregnated with 30 mg Levonorgestrel (Levonorgestrel, Beijing Zizhu Co., LTD) for 12 days, then intramuscular injection of 25 IU FSH (FSH, Ningbo Hormone Co., LTD) and 0.05 mg $PGF_{2\alpha}$ ($PGF_{2\alpha}$, Ningbo Hormone Co., LTD) at sponge withdrawal. All does were checked once daily to ensure that sponges remained in place during the treatment period (Baldassarre and Karatzas, 2004). Does that had lost their sponge were noted and the sponge was immediately replaced. Estrus was detected at 6 h intervals by visual observation (06:00, 12:00 and 18:00 h) during the 5 days following the end of treatment, using two intact teaser bucks fitted with an abdominal apron. Does were considered to be in estrus when standing to be mounted.

Intra-cervical insemination

Intra-cervical inseminations were performed by lifting the hindquarters of the doe over an elevated rail while the front legs remained standing on the ground. Each doe was inseminated within 4 h after semen preparation. The semen was deposited into the external opening of the first cervical fold, using a speculum inseminating pipette according to standard procedures (Salvador et al., 2005).

Intrauterine insemination

Intrauterine inseminations were performed on animals under local anaesthesia by injection of 4 ml procaine hydrochloride. Does were placed head down and supine on a 30° sloped surgical table with the hind legs tightly bound to the table in order to support most of the body weight after preparing for surgery. Intrauterine inseminations were performed as described (Vazquez et al., 2008; Wu et al., 2008). Briefly, after surgical preparation, the presence of mature preovulatory follicles was checked and the insemination needle with semen was inserted through the uterine horn. An extra volume of 0.5 ml of extender was used to wash out all the spermatozoa from the needle. Antibiotic cover was given by Tetracycline (20 mg/kg). Recovery was usually complete by 2-4 days post-treatment. Conception rates were determined by 25-day non-return rates.

PCR and Southern Blotting analysis

The genomic DNA from the kids was detected by screening using PCR technology with primers: forward primer (5′-CCCTTCCCTCGTCTCCAC-3′) and reverse primer (5′- CGTCGCCGATGGGTGGTGTTCT -3′). Each PCR sample consisted of 25 µl of solution containing 10-20 ng of template, 2.5 µl 10×Buffer, 2.5 µl $MgCl_2$, 2 µl dNTP, 0.5 µl of each primer, 0.5 µl Taq polymerase (TaKaRa). PCR consisted of 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 75 s, followed by 10 min extension at 72°C. Each PCR sample was subjected to electrophoresis on a 1% agarose gel.

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Table 2. Seminal parameters considered in the prescreening of sperm donors

Sperm donor	Ejaculate volume/ml	Motility rate/%	Acrosomal integrity rate/%	Abnormality rate/%	Sperm concentrations /10^9cells per ml	Live index
YW011	0.23±0.04	66.67±4.40	89.63±2.71	3.30±0.19^{a}	1.989±0.382	2.18±0.03^{A}
B2029	0.45±0.10	61.67±6.54	87.55±1.30	8.26±3.25^{b}	2.151±0.500	1.55±0.15^{B}

Means with different superscripts of capital letters in the same column are most significantly different ($p<0.01$); different superscripts of small letters mean significant difference ($p<0.05$).

DNA was separated electrophorectically on a 0.8% agarose gel and transferred to a nitrocellulose membrane. After air-drying, the DNA was cross-linked to the membrane by UV irradiation, and then hybridized with a biotinylated probe for the pEGFP-N_1 gene (Detector^{TM} PCR DNA Biotinylation Kit, KPL). Hybridization and washing procedures were as described previously (Zhao et al., 2008). Hybridization was carried out overnight at 42°C in a standard buffer solution (DNA Detector^{TM} HRP chemiluminescent Blotting Kit, KPL) containing 50 ng/ml of denatured probe. The washing and detection procedures were the same as described by Sambrook and Russell (2001).

Statistical methods

The mathematical model for kidding percentage was compared by one-way analysis of variance. Differences between means were tested for significance by the Duncan's new multiple range test (Weiss and Hassett, 1984). PCR and Southern blotting positive rates were analyzed using Yate's corrected X^2-test of independence and $p<0.05$ was considered statistically significant (Snedecor and Cohran, 1980). The seminal parameters of bucks were compared by t-test. The statistical software program SPSS 13.0 was used for all analyses.

RESULTS

Sperm quality and transfection efficiency

We selected two bucks, one Boer goat (B2029) and the other a Yudong White goat (YW011), as the sperm donors. The seminal parameters of the selected bucks are shown in Table 2. The differences in ejaculate volume, motility, acrosomal integrity and sperm concentration between the two bucks were not significant ($p>0.05$). However, the abnormality rate of B2029 was significantly higher than that of YW011 ($t = 2.772$, $p = 0.024$) and the live index was more significantly lower ($t = 6.299$, $p = 0.003$). Both sperm donors had a high ability to pick up exogenous DNA and no significant difference was found (64.09%±4.56% and 55.67%±1.77%, $t = 1.5118$, $p = 0.1910$).

Kidding results

The kidding results for different insemination deposition sites and sperm numbers are shown in Table 2. A total of 57 does kidded. There were 30, 20, 2 and 15 does kidded of Group I to Group IV, respectively. The kidding percentage was very significantly different among groups ($F_{3, 130} = 5.501$, $p = 0.001$) and it varied between 12.50% and 62.50%.

Transgenic kids produced by sperm mediation

A total of 76 kids were generated. The presence of the pEGFP-N_1 DNA was screened by PCR and also by Southern blotting analysis.The results of different groups are shown in Table 3. Eight kids were tested positive on the basis of agarose gel electrophoresis of the PCR-amplified fragment (Figure 1). Southern blotting analysis of the samples showed 5 positive kids (Figure 2).

DISCUSSION

We describe herein the possibility of SMGT producing transgenic goats. The results showed that transgenic goats could be produced efficiently with the method of artificial insemination by the sperm cell integration of the exogenous DNA. A total of 76 kids were generated, among them 5 kids tested positive by Southern blotting analysis. The major benefits of SMGT were found to be high efficiency, low cost and ease of use compared to other methods (Lavitrano et al., 1989; Lavitrano et al., 1992; Wall, 2002; Smith and Spadafora, 2005; Webster et al., 2005; Lavitrano et al., 2006; Li et al., 2006; Shen et al., 2006). Traditionally, the

Table 3. The results of does kidding, kids screened by PCR and Southern blotting analysis of different groups

Group	Does kidded	Kidding percentage/%	Number of Kids	Kids PCR positive	PCR positive rates/%	Kids Southern blotting positive	Southern blotting positive rates/%
I	30	62.50^{Aa}	42	4	9.52	2	4.76
II	10	34.48^{b}	11	1	9.09		
III	2	12.50^{B}	5	0			
IV	15	36.59^{b}	18	3	16.67	3	16.67

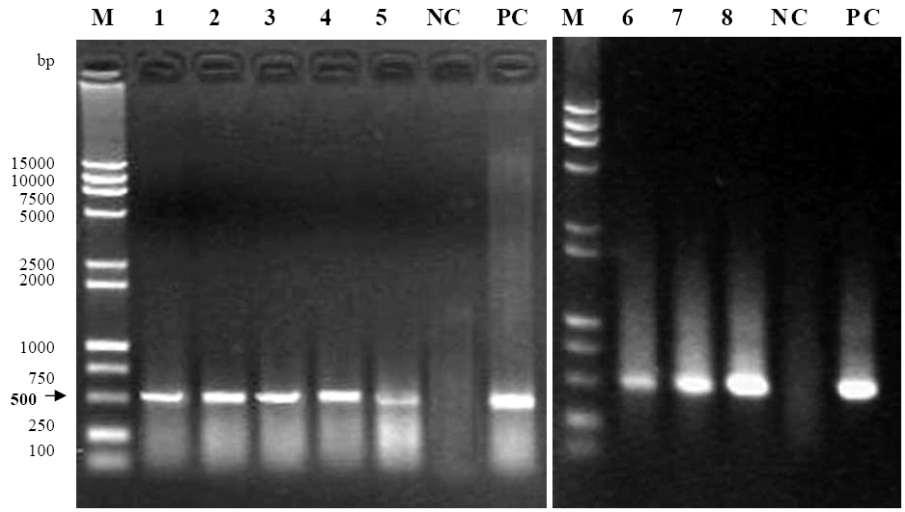

Figure 1. PCR analyses of genomic DNA samples extracted from the ears of eight transgenic kids. Lanes 1-8: the DNA extracted from the ears of eight transgenic kids. NC, negative control. PC, positive control.

production of transgenic animals has relied almost exclusively on the microinjection technique. The technique was first developed in mice, and the first batch of genetically engineered supermice was generated by applying the same technique (Gordon et al., 1980; Palmiter et al., 1983). However, that procedure **is** relatively complex, inefficient (with an efficiency of about 3%), and expensive (Wolf et al., 2000; Niemann et al., 2003; Lavitrano et al., 2006). When applied to other species these methods either work with lower efficiency or do not work at all. SMGT shows high efficiency in mice (Lavitrano et al., 1989; Maione et al., 1998). Subsequently, this technique was successfully adapted and optimized for use in large animals with high efficiency (Lavitrano et al., 2002; Lavitrano et al., 2003; Shen et al., 2006).

The key feature of this study is that we have obtained a number of transgenic goats with an efficiency of 6.58% by SMGT method. The selection of sperm donor also plays an important role in producing transgenic goats. Lavitrano demonstrated that there are two important parameters that must be optimal for the SMGT technique to be effective in swine: selection of sperm donor and optimization of DNA uptake (Lavitrano et al., 2003; Lavitrano et al., 2006). In our former study, we have found that different goat sperm donors appeared to differ in ability to bind foreign genes (Zhao et al., 2005). The standard sperm parameters, such as volume, concentration, presence of abnormal sperm, motility and the ability to bind foreign genes, were assessed one by one. We then selected two bucks, one Boer goat and another Yudong White goat, as sperm donors. We concluded that these two bucks could supply high quality semen and serve as good vectors for exogenous DNA.

Our study showed that frozen-thawed spermatozoa could also be used as a vector for introduction of exogenous DNA and produce transgenic goats. This is the first report of the SMGT method in this field. Anzar and Buhr (2006) found transfection efficiency of bull spermatozoa to be greater in frozen-thawed than in fresh spermatozoa, both from the same ejaculate. We have investigated the efficiency and the ability of fresh and frozen-thawed sperm to pick up exogenous DNA, and found that frozen-thawed goat sperm were more efficient and more reliable than fresh sperm cells (Zhao et al., 2008). The transgenic kid ratio was lower than the transgenic embryonic ratio, but freeze-

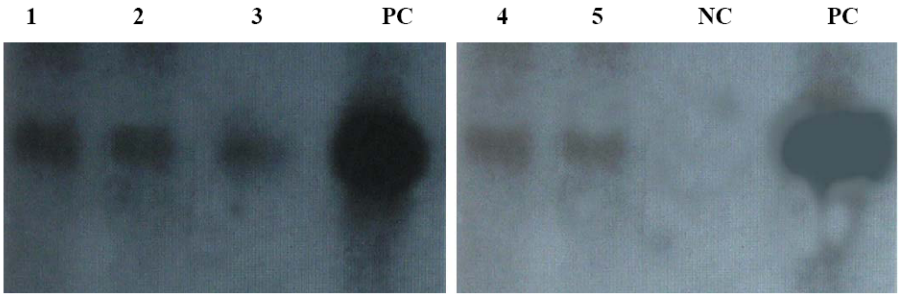

Figure 2. Southern blotting analyses of genomic DNA samples extracted from the ears of five transgenic kids. Lanes 1-5: the DNA extracted from the ears of five transgenic kids. NC, negative control. PC, positive control.

thawing treatment could enhance the efficiency of picking up exogenous DNA and producing transgenic kids. Due to low temperature and high salt concentration, semen cryopreservation reduced the integrity of sperm plasma membranes and enhanced sperm genomic DNA strand breakage significantly (Medeiros et al., 2002; Leboeuf et al., 2003; Purdy, 2006).

The data presented in this work demonstrate that intrauterine insemination may be suitable for reducing the number of frozen-thawed spermatozoa to one million and a gain in kidding percentage of 36.59%. Large doses of fresh semen are used in conventional artificial insemination. However, the number of spermatozoa can be greatly reduced if they are deposited close to the site of fertilization (Watson and Behan, 2002; Fantinati et al., 2005; Pelland et al., 2008; Vazquez et al., 2008), thus diminishing their loss along the uterine horns. In this work, the production of transgenic kids by SMGT is based upon the spontaneous uptake of exogenous DNA by goat spermatozoa removed from seminal plasma. A single standard dose for goat artificial insemination (AI) requires a huge amount of DNA which is expensive and time-consuming to obtain and purify. Also, the experimental bucks were housed in the University facilities, whereas the does were at goat farms far from the University. The long-term frozen storage of semen was very necessary for performing artificial insemination efficiently. In order to achieve a high kidding percentage with low numbers of frozen sperm, intrauterine deposition of semen is required. However, the kidding percentage in our study was not satisfactory. There are a number of possible reasons. The first is surgical laparotomy that results in distress for the does and in postoperative adhesions complicating either subsequent embryo production or delivery. The second is the low number of spermatozoa. The total number of spermatozoa inseminated per female is one of the main factors affecting fertility. It was found that 200 million frozen-thawed spermatozoa was sufficient to obtain acceptable kidding performance in conventional artificial insemination. Intrauterine insemination resulted in improved fertility rates and allowed a reduction in numbers of frozen-thawed spermatozoa inseminated to 5-20 million motile spermatozoa (Ritar et al., 1990). One million sperm could decrease significantly the amount of exogenous DNA required, but result in a low kidding percentage. The third is that the does were induced to estrus. Natural, synchronized or induced estrus can influence fertility. In our study, the does were synchronized. Despite improvements in the efficiency of hormonal synchronization of estrus, variability in the time of ovulation after treatment is an important impediment to attaining acceptable fertility after only one insemination with low number sperm. Goat intrauterine insemination with frozen goat semen should be optimized in the future.

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