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(ARTICLE)

Optimization of Transgenic Rabbit Embryo Production Using Pronuclear and Intracytoplasmic Gene Injection Techniques

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تحسين إنتاج أجنة الأرانب المعدّلة وراثيًا باستخدام تقنيتي الحقن الجيني داخل النواة الأبوية والحقن داخل السيتوبلازم

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Abstract:
This study compared three microinjection-based gene transfer methods intracytoplasmic sperm injection mediated transgenesis (ICSI-t), intracytoplasmic gene injection (ISI), and pronuclear injection (PNI) using a hyperactive piggyBac plasmid encoding green fluorescent protein (GFP). Female New Zealand White rabbits received 75 IU HMG for three consecutive days followed by 100 IU *hCG* to induce superovulation. Oocytes and zygotes were collected 15–16 hours later. Two DNA concentrations (10 and 20 ng/µl final) were tested for each method. Embryos cultured in TCM-199 with 15 mg/ml BSA at 38.5°C and 5% CO_2 were assessed at 24, 48, 72, 96, and 120 hours for cleavage, blastocyst formation, and GFP expression. The PNI 10 ng/µl group produced the highest blastocyst rate (42.1%, 16/38) with 37.5% (6/16) of those blastocysts GFP-positive. The PNI 20 ng/µl group gave fewer blastocysts (12.9%, 4/31) but a higher proportion expressed GFP (75.0%, 3/4). ICSI-t performed poorly, yielding blastocysts only at the lower concentration (11.1%, 1/9) with no GFP expression. ISI produced intermediate blastocyst rates (10 ng/µl: 11.3%, 6/53; 20 ng/µl: 10.3%, 4/39) but GFP expression in blastocysts only at the lower concentration (66.6%, 4/6). Higher DNA concentration consistently reduced embryo viability across all methods. Pronuclear injection at 10 ng/µl proved the most effective combination for generating transgenic rabbit embryos in vitro.

Keywords: **:** Rabbit transgenesis; pronuclear injection; intracytoplasmic sperm injection; piggyBac transposon; GFP expression; embryo culture.

الملخص
قارنت هذه الدراسة بين ثلاث طرائق لنقل الجينات قائمة على الحقن المجهري، وهي: النقل الجيني بوساطة حقن الحيوانات المنوية داخل السيتوبلازم (ICSI-t)، والحقن الجيني داخل السيتوبلازم (ISI)، والحقن داخل النواة الأبوية (PNI)، وذلك باستخدام بلازميد piggyBac فائق النشاط يرمّز للبروتين الفلوري الأخضر (GFP). تلقت إناث أرانب New Zealand White جرعة مقدارها 75 وحدة دولية من هرمون HMG لمدة ثلاثة أيام متتالية، تلتها جرعة مقدارها 100 وحدة دولية من هرمون hCG لتحفيز الإباضة الفائقة. جُمعت البويضات واللاقحات بعد 15–16 ساعة. وتم اختبار تركيزين من الحمض النووي لكل طريقة، وهما 10 و20 نانوغرام/ميكرولتر كتركيز نهائي. زُرعت الأجنة في وسط TCM-199 المحتوي على 15 ملغم/مل من BSA عند درجة حرارة 38.5°م وفي وجود 5% من ثاني أكسيد الكربون، ثم قُيّمت بعد 24 و48 و72 و96 و120 ساعة من حيث الانقسام، وتكوّن الكيسة الأريمية، والتعبير عن GFP. أظهرت مجموعة الحقن داخل النواة الأبوية بتركيز 10 نانوغرام/ميكرولتر أعلى معدل لتكوّن الكيسات الأريمية، إذ بلغ 42.1% (38/16)، وكانت نسبة 37.5% (16/6) من هذه الكيسات موجبة للتعبير عن GFP. أما مجموعة الحقن داخل النواة الأبوية بتركيز 20 نانوغرام/ميكرولتر فقد أعطت عددًا أقل من الكيسات الأريمية بنسبة 12.9% (31/4)، إلا أن نسبة أعلى منها عبّرت عن GFP وبلغت 75.0% (4/3). وقد أظهر أسلوب ICSI-t أداءً ضعيفًا، حيث لم يُنتج كيسات أريمية إلا عند التركيز الأقل بنسبة 11.1% (9/1)، دون تسجيل أي تعبير عن GFP. أما طريقة ISI فقد أعطت معدلات متوسطة لتكوّن الكيسات الأريمية، بلغت 11.3% (53/6) عند تركيز 10 نانوغرام/ميكرولتر و10.3% (39/4) عند تركيز 20 نانوغرام/ميكرولتر، إلا أن التعبير عن GFP في الكيسات الأريمية ظهر فقط عند التركيز الأقل بنسبة 66.6% (6/4). وبوجه عام، أدى ارتفاع تركيز الحمض النووي إلى انخفاض حيوية الأجنة في جميع الطرائق المستخدمة. وقد تبيّن أن الحقن داخل النواة الأبوية بتركيز 10 نانوغرام/ميكرولتر يمثّل التركيبة الأكثر فعالية لإنتاج أجنة أرانب معدّلة وراثيًا في المختبر.

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الكلمات المفتاحية: التوليد الجيني في الأرانب؛ الحقن داخل النواة الأوّلية؛ الحقن المجهري للحيوان المنوي داخل سيتوبلازم البويضة؛ عنصر النقل الجيني القافز ؛ زراعة الأجنّة.

1. Introduction

Transgenic rabbits have an outstanding and important role in biomedical research because of their physiological features and similarity to human pathogenic processes when contrasted with conventional rodent models. While the lipoprotein metabolism of the mouse is characterized by high-density lipoproteins, rabbits show cholesterol metabolism based on low-density lipoproteins, like humans. For this reason, these animals are used extensively in research into atherosclerosis, heart problems, and other metabolic disorders (Wang et al., 2013; Matsuhisa et al., 2020). The close evolutionary relationship between rabbits and humans, coupled with some practical factors such as short gestation (31-32 days), relatively large litters (7-9 pups), early maturity, and manageable cost of breeding has allowed transgenic rabbits to become increasingly significant as animal models for biomedical research (Xu et al., 2021; Yang et al., 2019). Thus, many animal models of diseases related to humans, such as cardiovascular conditions, immunodeficiencies, eye disorders, oncologic diseases, and infectious conditions have been developed using transgenic rabbits (Song et al., 2017; Yuan et al., 2017; Xu et al., 2021). Apart from the development of disease models, rabbits were found to be useful bioreactors for producing recombinant protein-based medications. The successful commercialization of recombinant human C1 esterase inhibitor (Ruconest®), produced in the milk of transgenic rabbits, highlights their significant potential in pharmaceutical biotechnology (Matsuhisa et al., 2020).

Nevertheless, the creation of transgenic rabbits is technologically complicated and not very efficient. One of the most frequently used technologies for obtaining transgenic animals is pronuclear microinjection. Transgenesis in rabbits implies direct transfer of foreign DNA into one of the pronuclei of fertilized zygotes and has been effectively used for the generation of many transgenic strains (Hirabayashi et al., 2000; Chrenek et al., 2005; Hrenek, 2017; Zhmool, et al., 2026). However, it requires careful examination and manipulation of pronuclei and is thus quite difficult and technologically challenging, and the variability in the efficiency of DNA integration, embryo viability, and expression of the transgene further reduces its efficiency (Chrenek *et al.*, 2005; Garcia, 2018). Even though rabbit pronuclei are relatively easy to examine and manipulate in comparison with livestock such as cattle and pigs, effective microinjection is still complicated and requires special training and equipment (Garcia, 2018).

An alternative strategy involves intracytoplasmic sperm injection-mediated transgenesis (ICSI-T), which exploits the ability of spermatozoa to bind and transport exogenous DNA into oocytes. In such an approach, spermatozoa are incubated with heterologous DNA prior to their introduction directly into mature eggs via intracytoplasmic sperm injection procedure. This method has already proven its effectiveness in various animals and can be considered as a possible replacement for pronuclear injection procedures (Moisyadi et al., 2009). Rabbit studies involving the use of sperm-mediated gene transfer and ICSI-associated transgenesis procedures revealed that genetically modified embryos/offsprings can be obtained using these techniques (Vasicek et al., 2010; Li et al., 2010). At the same time, the efficiency and reproducibility of the mentioned methods have proved to vary across different species because of the specificities associated with the characteristics of spermatozoa in question as well as with the mechanisms of DNA uptake and integration (Moisyadi et al., 2009; Vasicek et al., 2010; Aloraibi,2025).

Intracytoplasmic gene injection (ICGI) is a third microinjection technique where DNA is injected directly into the ooplasm without the need for pronuclear recognition. Such techniques might simplify the process of pronuclear handling and increase the chances of survival. Despite the successes of cytoplasmic injection in various mammals, very few works describe such procedures for use on rabbit oocytes (Garcia, 2018). Comparative experiments between pronuclear injections and cytoplasmic gene injections under identical conditions are very rare, thus making it impossible to define which procedure is the best one for rabbits.

The advent of genome editing techniques has greatly increased the opportunities for the generation of genetically modified rabbits. The development of novel transgenic techniques like transcription activator-like effector nucleases (TALENs), CRISPR/Cas9, and transposon-based vectors is expected to provide more precise and effective means of manipulating the genomes of rabbits compared to traditional methods

(Song et al., 2013; Song et al., 2020; Xu et al., 2021). Of all the new techniques mentioned, piggyBac transposon-based technique has been gaining popularity in the recent past due to its ability to ensure effective and stable genome modification by the cut-and-paste transposition process. Contrary to plasmid-mediated transgenics, piggyBac transposon-based transgenics offers higher efficiency of genomic integration and maintenance of transgene expression (Song et al., 2020; Yang et al., 2019). The continued evolution of rabbit transgenesis and animal pharming is further evidence of the need for enhanced methods of gene delivery and integration to optimize transgene transfer and expression efficiency (Song et al., 2025; Matsuhisa et al., 2020;Lamma, et al., 2019).

Despite progress made in the field of rabbit transgenesis, a comparative study on pronuclear injection, intracytoplasmic sperm-mediated gene transfer, and intracytoplasmic gene injection with identical genetic constructs is not yet available. Determining the relative efficiency of these techniques is essential for optimizing transgenic rabbit production and expanding the use of rabbits as models for biomedical research and biopharmaceutical production. Therefore, the present study aimed to optimize transgenic rabbit embryo production by systematically comparing pronuclear and intracytoplasmic gene injection techniques and evaluating their effectiveness for transgene delivery and embryo development.

The present study therefore set out to determine, first, which of the three methods 'ICSI-t, ISI, or PNI' supports the highest rates of rabbit embryo development to the blastocyst stage after gene transfer; second, how DNA concentration (10 versus 20 ng/µl) affects embryo survival and transgene expression; and third, what combination yields the highest proportion of GFP-expressing blastocysts. The findings establish a baseline for optimizing transgenic rabbit production and inform method selection for future biopharmaceutical and disease modeling work.

2. Literature Review

Transgenesis techniques for generating transgenic rabbits have undergone substantial development over the last few decades as part of more general developments in reproductive biotechnology and genome engineering. Early efforts in transgenesis experiments made use of pronuclear microinjection, a process that had advantages of foreign DNA direct introduction into the embryo but was characterized by low efficiency and high rates of embryonic loss. Hirabayashi et al. (2000) developed an approach for overcoming a technical problem inherent to rabbit zygotes by adding centrifugation as a preliminary stage prior to microinjection. Despite the fact that the new technique helped increase efficiency of the procedure, stable rates of DNA transgene integration were still rather low because of biological limitations inherent to this approach and based on the principle of random insertion of the gene. In turn, the results of Chrenek et al. (2005) showed that simultaneous microinjection of DNA into both pronuclei significantly enhanced transgene integration rates compared to conventional techniques involving a single pronucleus.

The drawbacks in the technique of microinjection of pronuclei prompted the search for new ways of introducing genes. The ICSI-mediated transgenesis method appeared to be one such technique due to the reason that this approach integrates fertilization and DNA introduction into one step. According to Moisyadi et al. (2009), this method may lead to a decrease in mechanical injury to embryos, while at the same time increasing the efficiency of DNA insertion. Indeed, as has been demonstrated by experiments conducted by Li et al. (2010), it is possible to produce transgenic rabbits using this technique. However, comparison of the techniques shows that although ICSI is more efficient in DNA insertion, transgene introduction still depends on random recombination, leading to inefficient transgenesis. Sperm-mediated gene transfer has also been shown to be a less invasive way of introducing genes (Vasicek et al., 2010). Unfortunately, lack of consistency in results hinders further application of this technique.

One key point in the transgenesis in rabbits was reached with the development of methods for targeted genome editing. While traditional methods involve random genome integration, genome editing tools allow scientists to perform precise changes at certain genomic locations. According to Song et al. (2020), ZFN, TALEN, and CRISPR/Cas9 technologies have improved accuracy while minimizing the uncertainty in previous technologies used. An important development using TALENs technology has been the generation of immunodeficient rabbits lacking RAG1 and RAG2, since the use of such technology has

enabled the creation of rabbit models through precise gene editing rather than random transgenesis (Song et al., 2013). Additionally, embryo transfer has made it possible to increase efficiency through multiplex gene targeting (Song et al., 2017).

Of the various editing techniques that exist, CRISPR/Cas9 has emerged as the most dominant technique owing to its ease of use and effectiveness. For example, Yuan et al. (2017) successfully managed to induce the creation of congenital cataracts by knocking out the αA-crystallin gene, thus proving that the use of rabbits in human hereditary disease research is possible. Additionally, Xu et al. (2021) noted that edited gene rabbits fill a translational niche between rodents and other mammals because they resemble humans physiologically. This idea is backed up by the use of specific rabbits, such as those carrying a human apolipoprotein A-II transgene, to study atherosclerosis and lipids (Wang et al., 2013). Finally, all mentioned articles demonstrate that rabbits gain significance as translational medicine models instead of being transgenic animals employed for concept proving experiments.

On the other hand, recently some reviews highlighted that modern technology development for rabbits undergoes a paradigm shift toward precise genome editing and production of biologics. According to Matsuhisa et al. (2020), further advancements would rely on the combination of reproductive technologies with genome-engineering techniques to increase efficiency and repeatability. This prediction can be explained by the emergence of high-yielding rabbit strains specialized for the pharming of animals that involve the incorporation of modified genes alongside productive characteristics (Song et al., 2025). However, even though there has been considerable advancement in this field, certain issues still exist, including low efficiency, off-target effects, embryo viability, and scalability. Thus, improvements in embryo manipulation protocols and gene transfer techniques are needed.

Generally, the literature shows a clear advancement in transgenesis from techniques of random integration to genome editing technologies. Although much progress has been made, embryo manipulations, transgene delivery, and embryo competency are still regarded as important bottlenecks. Consequently, research works aimed at optimizing embryo injections and the assessment of the development of embryos resulting from injection procedures are still important towards improving rabbit transgenesis.

The lack of data on concentration-response is another important issue in the literature. Studies testing only one dose without rationale were the norm in transgenic studies. Only Marh et al. (2012) gave dose-response data using mouse embryos in which doses between 10 and 20 ng/µl resulted in about 90% reduction in live births. It is unclear whether rabbits share the same susceptibility. Additionally, there is no comparison of the optimal dose of DNA in PNI, ISI, and ICSI-t. The current study will fill this void.

There were few factors which motivated me to conduct the current study. Firstly, there is a lack of direct comparison between PNI, ISI, and ICSI-t for transgene delivery in rabbit embryos using the same experimental setup. Any available comparative studies are done by either comparing two techniques or have used varying transgenes, concentrations, or culture media systems. Secondly, the dose-response relationship of the effect of DNA concentration on embryo viability in rabbits is yet unknown. In other words, researchers lack evidence-based basis to choose optimal injection concentrations.

Firstly, it was intended to discover the combination of procedure (PNI, ISI, or ICSI-t) along with the DNA concentration (10 ng/µl or 20 ng/µl), which provides the best blastocyst development and green fluorescent protein expression in rabbit embryos. Additionally, secondary goals were to define the toxic impact of high DNA concentration on all procedures and determine developmental rates for each of them.

3. Methodology

3.1 Experimental Design

The study employed a 3 × 2 factorial design: three gene transfer methods (ICSI-t, ISI, PNI) crossed with two final DNA concentrations (10 ng/µl and 20 ng/µl). A total of 17 female New Zealand White rabbits served as oocyte or embryo donors (8 for ISI/PNI experiments, 9 for ICSI-t experiments). Each rabbit contributed oocytes or zygotes to only one experimental condition.

3.2 Animals and Housing

Sexually mature New Zealand White rabbits (5–7 months old, weighing 3.5–4.5 kg) were housed individually at the Department of Animal Production, Faculty of Agriculture, University of Tripoli, Libya. The animals were maintained under controlled environmental conditions, with a room temperature of 18-21°C and a photoperiod of 10 h light and 14 h dark. Standard rabbit feed and fresh water were provided ad libitum throughout the experimental period. All animal handling and experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals.

Figure 1. Individually housed sexually mature New Zealand white rabbits maintained under controlled environmental conditions for transgenesis experiments

3.3 Superovulation and Ovulation Induction

Donor female New Zealand White rabbits maintained at the Department of Animal Production, Faculty of Agriculture, University of Tripoli, Libya, were superovulated using 75 IU human menopausal gonadotropin (Livzon Pharmaceutical Group Inc., Zhuhai, China) administered subcutaneously once daily for three consecutive days at 24-hour intervals. Twenty-four hours after the final HMG injection, ovulation was induced by intravenous administration of 100 IU human chorionic gonadotropin (Pregnyl®, Organon, Netherlands). For embryo collection, females were naturally mated immediately after hCG administration with fertile males of proven reproductive performance. For oocyte collection, ovulation was induced without fertilization by applying mechanical vaginal stimulation using a sterile cotton swab several times before and after hCG injection.

3.4 Sperm Collection and Preparation

Spermatozoa for ICSI-t were collected from fertile males using an artificial vagina. Males were trained for one week before the experiment. Freshly collected semen was assessed macroscopically and microscopically, then transferred to a microcentrifuge tube and centrifuged at 2000 rpm for 5 minutes to remove the gel fraction. The pellet underwent swim-up in HSOF medium supplemented with 4 mg/ml BSA for 30 minutes at 38.5°C. The upper fraction (3 ml) was collected, centrifuged at 1500 rpm for 5 minutes, and resuspended in HSOF with 4 mg/ml BSA to a final concentration of 1×10^6 spermatozoa/ml. The suspension was held at 4°C for 1 hour, then mixed 1:1 with the DNA construct (at either 20 ng/µl or 40 ng/µl pre-mix concentration, yielding 10 or 20 ng/µl final after mixing) and incubated for an additional 10 minutes at 4°C before immediate use in ICSI-t.

3.5 Gene Construct

The hyperactive piggyBac plasmid pmhyGENIE-3-RNA-OUT (Figure 1) carried a green fluorescent protein (GFP) expression cassette along with the piggyBac transposase and transposon elements in a single construct. Stock concentration was 511 ng/µl stored at –20°C. Working dilutions were prepared using embryo-tested water (ETS, Sigma W1503). For 10 ng/µl final concentration: 10 µl of stock was diluted with 245.5 µl ETS to 20 ng/µl intermediate, then mixed 1:1 with either PVP (for ISI and PNI) or sperm suspension (for ICSI-t). For 20 ng/µl final: 2.5 µl stock diluted with 29.5 µl ETS to 40 ng/µl intermediate, then mixed 1:1 as above.

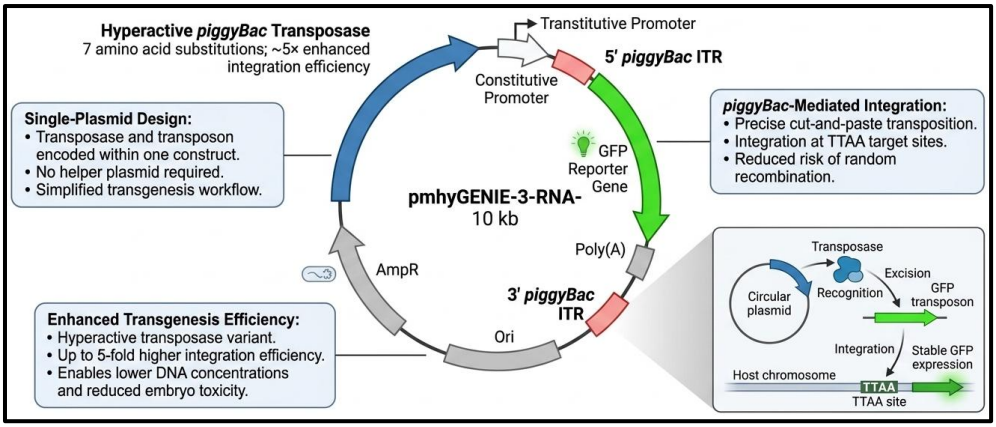

Figure 2. Map of the pmhyGENIE-3-RNA-OUT plasmid. (Adapted from Marh et al., 2012; original construct provided by S. Moisyadi, University of Hawaii.)

The piggyBac system offers two advantages over conventional plasmids. First, the transposase mediates precise cut-and-paste integration rather than random illegitimate recombination, potentially reducing insertional mutagenesis risk. Second, the hyperactive transposase variant (containing seven amino acid substitutions) increases integration efficiency up to five-fold compared to wild-type. For rabbit transgenesis, this means lower DNA concentrations may achieve acceptable integration rates, reducing embryo toxicity (Figure 1).

3.6 Oocyte and Embryo Collection

Fifteen to sixteen hours after hCG administration, donor animals were anesthetized with intramuscular ketamine hydrochloride (30 mg/kg, Ketamine, Brema, Germany) and xylazine hydrochloride (5 mg/kg, Basilazin, Bavet, Germany). Laparotomy was performed via midline incision. The number of corpora lutea on each ovary was recorded. Oviducts were flushed with Dulbecco's PBS containing 10% fetal bovine serum using a blunt cannula inserted near the uterotubal junction. Flush medium (5–10 ml per oviduct) was collected into 100 mm Petri dishes via a plastic cannula placed in the fimbria.

For oocyte collection (ICSI-t groups): recovered oocytes were transferred to HSOF containing 0.1% hyaluronidase and vortexed for approximately 2 minutes to remove cumulus cells. Denuded oocytes with smooth, granule-free vitellus, intact zona pellucida, and visible first polar body were selected for ICSI-t.

For embryo collection (ISI and PNI groups): recovered embryos were treated similarly with 0.1% hyaluronidase in HSOF but vortexed for only 1 minute. Embryos at the pronuclear stage (visible pronuclei under stereomicroscope) were assigned to PNI; those without visible pronuclei but showing two polar bodies were assigned to ISI.

3.7 Microinjection Procedures

All microinjections were performed using a Piezo-electric actuated micromanipulator to minimize oocyte/embryo damage.

ICSI-*t*: Spermatozoa pre-incubated with DNA were drawn tail-first into an injection pipette (6–7 µm diameter). The oocyte was positioned with the polar body at 6 or 12 o'clock. The pipette entered the vitellus at approximately 3 o'clock, and a single spermatozoon was released near 9 o'clock.

ISI: DNA-PVP mixture (10 or 20 ng/µl final concentration) was injected into the ooplasm of pronuclear-stage embryos using micropipettes with 0.5–2 µm inner diameter. Successful injection was confirmed by slight swelling of the vitelline membrane.

ISI avoids the need to target the pronucleus, which is technically easier but delivers DNA to the cytoplasm where it may be degraded by nucleases before reaching the nucleus. The piggyBac transposase, if expressed from the same plasmid, must be translated in the cytoplasm and then enter the pronucleus to act. This extra step may explain why ISI showed lower blastocyst rates than PNI in our study (Figure 3).

PNI: DNA-PVP mixture was injected directly into one pronucleus of pronuclear-stage embryos using the same pipette specifications. Pronuclear expansion following injection confirmed successful delivery.

PNI deposits DNA directly into the transcriptionally active male pronucleus. In rabbits, zygotic genome activation occurs around the 8–16 cell stage (later than in mice), but the pronucleus already contains all the machinery for DNA replication and repair. Direct nuclear delivery may protect the transgene from cytoplasmic nucleases and place it closer to the integration machinery. The visible pronuclear expansion after injection serves as a real-time quality control step-absence of expansion suggests the DNA missed the pronucleus or leaked out (Figure 4).

Following injection, embryos from all groups were transferred to 35 mm Petri dishes containing TCM-199 medium with 15 mg/ml BSA and allowed to recover for 1 hour at 38.5°C in 5% CO_2. Lysed embryos were discarded.

Figure 3 provides a direct visual comparison of the three microinjection techniques used in this study. Each row corresponds to one method: (a) ICSI-t, (b) ISI, and (c) PNI. For each method, the left image shows the oocyte or zygote before injection, and the right image shows the same embryo immediately after successful delivery of the DNA-containing solution (or DNA-incubated spermatozoon, in the case of ICSI-t). The Piezo-electric actuator minimized cytoplasmic deformation in all cases.

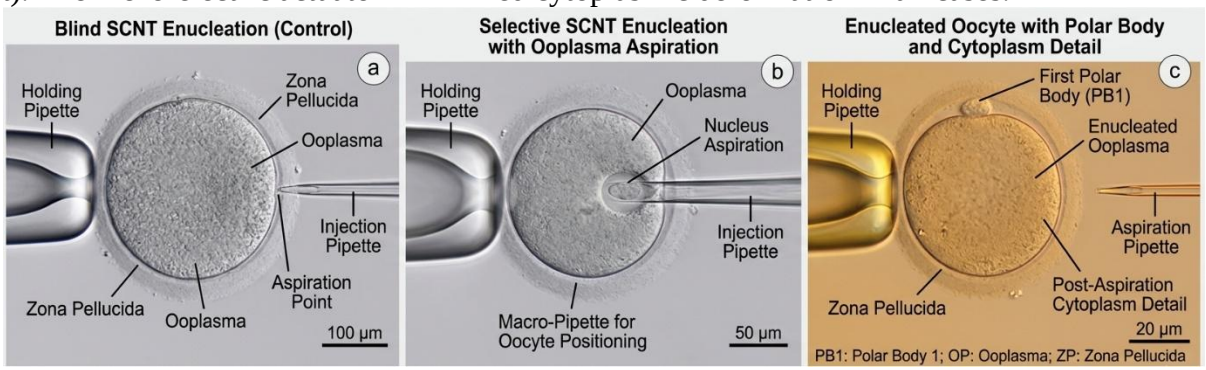

Figure 3. Comparison of transgene delivery techniques in rabbit embryos. (a) Intracytoplasmic sperm injection-mediated transgenesis (ICSI-t) (b) Intracytoplasmic gene injection (ISI) (c) Pronuclear injection (PNI) (with original images).

3.8 *In Vitro* Culture

Recovered embryos were cultured in Tissue Culture Medium-199 (TCM-199; Gibco, Thermo Fisher Scientific, USA) supplemented with 15 mg/mL bovine serum albumin (BSA), 1000 IU/mL penicillin-G, and 100 µg/mL streptomycin sulfate. Embryos were incubated at 38.5°C in a humidified atmosphere containing 5% CO_2 in air. Prior to use, the culture medium was adjusted to a pH of 7.3–7.4 and an osmolarity of 270–275 mOsm/kg. Embryonic development was monitored at 24, 48, 72, 96, and 120 h post-microinjection. At each evaluation time point, cleavage rate and developmental stage (2-cell, 4-cell, morula, blastocyst, and expanded blastocyst) were recorded. Beginning at 72 h post-microinjection, green fluorescent protein (GFP) expression was assessed using an epifluorescence microscope equipped with the appropriate excitation and emission filters. Representative images were captured for qualitative assessment of transgene expression.

3.9 Statistical Analysis

Data were analysed using SPSS 13.0. Analysis of variance (ANOVA) with post-hoc comparisons was used for intergroup differences in cleavage and blastocyst rates. The Mann-Whitney U test compared DNA concentration effects within each method. Differences were considered significant at $p < 0.05$.

4. Results

4.1. Superovulation Outcomes

Eight rabbits assigned to ISI and PNI experiments yielded 348 corpora lutea and 25 follicles, giving an ovulation rate of 93.3% (calculated as corpora lutea divided by total corpora lutea plus follicles). Oviductal flushing produced 328 embryos. Four embryos were of poor quality and one was unfertilized; these were excluded. After denudation, 261 embryos with confirmed fertilization (two polar bodies) were available for injection—108 assigned to PNI and 153 to ISI, though final injected numbers were 106 for PNI and 155 for ISI due to some embryos failing to survive denudation.

Nine rabbits assigned to ICSI-t experiments yielded 225 corpora lutea and 44 follicles (ovulation rate 83.6%). Flushing produced 100 oocytes, none of which were of poor quality. Seven oocytes were lost during denudation, leaving 93 oocytes for ICSI-t injection.

Table 1 summarizes superovulation results. The mean number of corpora lutea per rabbit differed substantially between the ISI/PNI group (43.5 ± 9.9) and the ICSI-t group (25.0 ± 4.2), reflecting individual variation in superovulation response rather than systematic differences between experimental arms.

Table 1. Superovulation results across experimental groups

Parameter	ISI/PNI experiments	ICSI-t experiments
Number of rabbits	8	9
Total corpora lutea	348	225
Mean CL per rabbit	43.5 ± 9.9	25.0 ± 4.2
Total follicles	25	44
Ovulation rate (%)	93.3	83.6
Total oocytes/embryos recovered	328	100
Usable after denudation	261	93

4.2. Embryo Survival, Cleavage, and Blastocyst Formation

In vitro developmental results are shown in Table 2. There was significant variation in lysis and degeneration among the different groups (ANOVA, $p < 0.05$). The high degeneration value belonged to the ICSI-t at 20 ng/µl group (41.7%, 20/48), whereas the low degeneration value occurred for the PNI at 20 ng/µl group (5.8%, 3/52). On the other hand, degeneration was higher with high concentration than low concentration within the ICSI-t treatment (41.7% vs 24.4%), and the reverse happened with the ISI and PNI treatments (12.8% vs 10.4%; 13.0% vs 5.8%).

The cleavage rates (percentage of surviving embryos which divided at least once in 24 hours) also varied among the groups. The highest cleavage rate was observed with PNI 10 ng/µl (80.8%, 38/47) followed by ISI 10 ng/µl (77.9%, 53/68). The lowest cleavage rate was found with ICSI-t 10 ng/µl (26.5%, 9/34). The concentrations of 20 ng/µl reduced the cleavage rate as compared to the lower concentrations within each method, except for ICSI-t where a higher cleavage was observed at the higher concentration (35.7% vs 26.5%). However, this difference was not statistically significant.

A much greater variation in the blastocyst rate was observed among the groups. The blastocyst rate was highest with PNI 10 ng/µl (42.1%, 16/38). PNI 20 ng/µl showed fewer blastocyst (12.9%, 4/31) as compared to PNI 10 ng/µl and the difference was statistically significant (Mann-Whitney U, $p<0.05$). ISI generated blastocyst rates were 11.3% (lower concentration) and 10.3% (higher concentration), showing no significant effect of concentration on blastocyst rates. The blastocyst rate for ICSI-t was

Table 2. In vitro development and GFP expression outcomes

Method	Conc (ng/µl)	Embryos injected	Degenerated n (%)	Surviving embryos	Cleaved n (%)	GFP+ among cleaved n (%)	Blastocysts n (% of cleaved)	GFP+ blastocysts n (%of blastocysts)
ICSI-t	10	45	11 (24.4)	34	9 (26.5)	0 (0)	1 (11.1)	0 (0)
ICSI-t	20	48	20 (41.7)	28	10 (35.7)	3 (30.0)	0 (0)	—
ISI	10	78	10 (12.8)	68	53 (77.9)	20 (37.7)	6 (11.3)	4 (66.6)
ISI	20	77	8 (10.4)	69	39 (56.5)	18 (46.2)	4 (10.3)	0 (0)
PNI	10	54	7 (13.0)	47	38 (80.8)	13 (34.2)	16 (42.1)	6 (37.5)
PNI	20	52	3 (5.8)	49	31 (63.3)	15 (48.4)	4 (12.9)	3 (75.0)

Figure 4 illustrates the morphological quality of oocytes and embryos before microinjection. Panel (A) shows mature cumulus-oocyte complexes collected for ICSI-t. Panel (B) displays denuded oocytes (for ICSI-t) and denuded zygotes (for

ISI/PNI) after hyaluronidase treatment; arrows indicate the first polar body. Panel (C) presents a fertilized embryo with two visible pronuclei and two polar bodies, the typical stage for ISI and PNI.

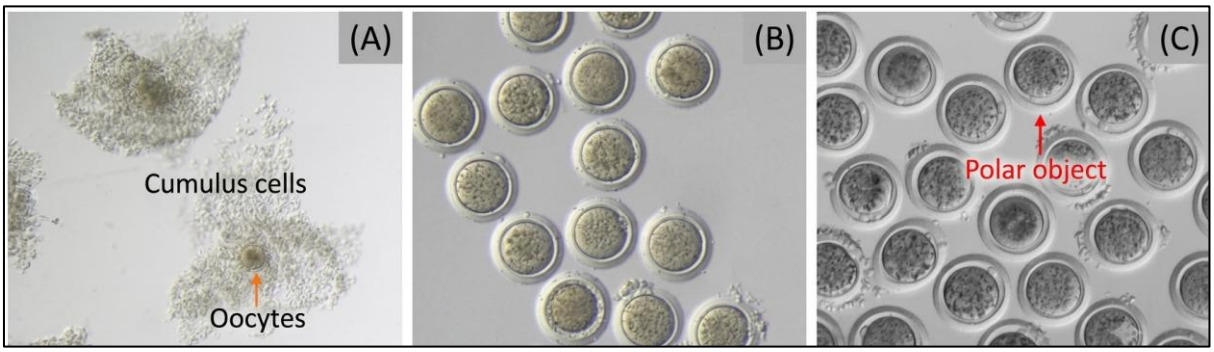

Figure 4. Morphological characteristics of rabbit oocytes and fertilized embryos used for gene transfer procedures**.** (A) Mature oocytes before denudation, still surrounded by cumulus cells. (B) Denuded oocytes (left, for ICSI-t) and denuded zygote (right, for ISI/PNI); arrows indicate the first polar body. (C) Fertilized two-pronuclear embryo (arrows point to pronuclei) with two polar bodies, employed for ISI and PNI. (Original micrographs.)

4.3. Embryo Morphology and GFP Expression

Figure 5 is a demonstration of embryos from each experimental group at certain developmental stages in brightfield and fluorescence microscopy.

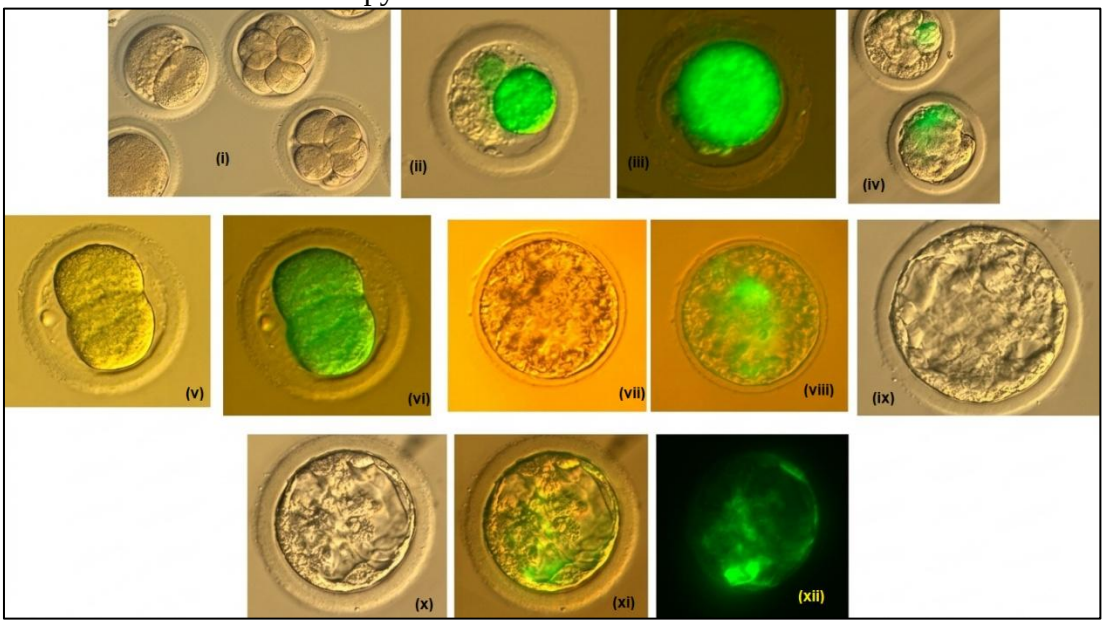

Figure 5. Representative preimplantation developmental stages and GFP expression in rabbit embryos following ICSI-t, ISI, and PNI-mediated transgene delivery. (i) Cleaved embryo (PNI, 10 ng/µl ; (ii) Degenerated embryo showing GFP expression from the ICSI-t 20 ng/µl group; (iii) Degenerated embryo showing GFP expression from the PNI 20 ng/µl group; (iv) Two-cell embryo from the ICSI-t 20 ng/µl group under brightfield microscopy.; (v) Corresponding GFP fluorescence image of the embryo shown in (d); (vi) Morula-stage embryo from the ICSI-t 20 ng/µl group showing GFP expression; (vii) Expanding blastocyst from the PNI 10 ng/µl group under brightfield microscopy; (viii) Corresponding GFP fluorescence image of the embryo); (ix) Blastocyst-stage embryo from the ISI 10 ng/µl group.; (x) Blastocyst-stage embryo from the PNI 20 ng/µl group under brightfield microscopy; (xi) Combined brightfield and fluorescence image of the embryo; (xii) GFP fluorescence image of the same blastocyst.

Detection of green fluorescence protein (GFP) in degenerated embryos may be due to passive diffusion or leakage through compromised membranes. The ICSI-t (20 ng/µl) group resulted in the formation of GFP-positive two-cell and morula stages but not blastocysts, implying an inhibitory effect on development depending on the DNA concentration although the DNA is successfully introduced into the embryos. In the PNI (10 ng/µl) group, GFP was uniformly detected in the blastocyst stage and implies that the

integration of genes occurred before the first cleavage with the production of non-mosaic GFP expression. In the PNI (20 ng/µl) group, mosaic expression of GFP-positive blastocysts was observed, which implies a delay in gene integration after one or more cell division. The same result was obtained for the ISI (10 ng/µl) group except with reduced developmental efficiency.

4.4. GFP Expression Patterns

GFP expression appeared as early as the 2-cell stage in some embryos, though most positive signals emerged by the morula stage (72–96 hours). Among blastocysts, GFP expression rates differed markedly (Table 2). PNI 20 ng/µl showed the highest proportion of GFP-positive blastocysts (75.0%, 3/4), followed by ISI 10 ng/µl (66.6%, 4/6) and PNI 10 ng/µl (37.5%, 6/16). No blastocysts in the ICSI-t groups expressed GFP, and the single blastocyst in the ICSI-t 10 ng/µl group was negative.

Notably, the relationship between DNA concentration and GFP expression among blastocysts inverted between PNI and ISI. For PNI, higher concentration produced a higher proportion of GFP-positive blastocysts despite reducing total blastocyst yield. For ISI, GFP expression in blastocysts disappeared entirely at the higher concentration, though expression in earlier-stage embryos remained detectable (46.2% of cleaved embryos at 20 ng/µl expressed GFP, but none reached blastocyst with expression).

The major results included the following: first, PNI with 10 ng/µl resulted in the highest blastocyst rate (42.1%); second, PNI with 20 ng/µl resulted in the highest rate of GFP positive blastocysts (75.0%) although significantly lower number of blastocysts was generated; third, ICSI-t performed badly at both concentrations, resulting in a single blastocyst with no GFP expression; fourth, ISI also resulted in an intermediate number of blastocysts, however, there was a concentration-dependent loss of GFP expression within blastocysts with high concentration; fifth, high DNA concentration negatively affected embryo viability in all techniques, although the effect differed, being the highest for ICSI-t, moderate for PNI and least for ISI; sixth, neither technique optimized both parameters; thus, PNI 10 ng/µl gave the highest yield, while PNI 20 ng/µl gave the highest GFP positive rate.

5. Discussion

This current study is the first one directly comparing the effectiveness of PNI, ICSI-T, and IGI in producing transgenic rabbit embryos by employing a highly active piggyBac transposon vector at two different DNA concentrations. The results indicate that not only the type of DNA delivery system but also DNA concentration affects embryo development and transgene expression. These results further add to the existing studies focused on enhancing the efficiency of rabbit transgenesis, which continues to be a difficult task even with the current advanced genome editing technology (Song et al., 2020; Xu et al., 2021).

Out of all the techniques tested, pronuclear injection was identified as the most suitable technique for the support of embryo development and transgene expression. Blastocyst formation was highest at 10 ng/µl with PNI compared to IGI and ICSI-T. These results are in line with other research studies that successfully achieved transgenic rabbits using PNI (Hirabayashi et al., 2000; Chrenek et al., 2005; Hrenek, 2017). Pronuclear injection is used most commonly as the technique of choice to produce transgenic rabbits since it allows DNA to be directly injected into the nucleus. It is known that pronuclear injection increases the chances of genetic modification by delivering DNA to the cell before the activation of embryonic genome function. The effectiveness of pronuclear injection seen in this study indicates that being close to replication and transcription of the nucleus may enhance the integration efficiency of the gene. Similarly, this concept is supported in research on the production of transgenic mice and rabbits where pronuclear localization helped improve gene expression (Hirabayashi et al., 2000; Yang et al., 2019).

The slightly reduced developmental ability after IGI shows that cytoplasmic introduction of foreign genes is an inefficient mode of transgenic incorporation. While IGI removes the problem associated with the observation of the pronucleus, any DNA transferred into the cytoplasm needs to withstand its destruction and transport into the nucleus for integration. As mentioned by Garcia (2018), the methods used for embryo manipulation by cytoplasmic introduction of DNA often show variability because of the differences in trafficking and transport within cells. The low blastocyst formation rate seen in the current

experiment supports this conclusion and indicates that further work on DNA introduction is necessary to make IGI as efficient as PN injection in rabbits.

Poor efficiency of ICSI-based transgenesis was an important issue. While ICSI-T has produced successful outcomes in creating transgenic embryos and offspring in different animals (Moisyadi et al., 2009), its implementation in rabbits has shown inconsistent results. Previous studies have proven that sperm-mediated gene delivery and ICSI-based transgenesis are possible in rabbits (Vasicek et al., 2010; Li et al., 2010); however, very low levels of blastocyst production, as well as complete absence of GFP-containing blastocysts with higher DNA concentrations were detected in the present investigation. These results imply the fact that rabbit sperm cells could be different from other species' sperm cells in terms of binding and carrying exogenous DNA molecules. The differences between species in chromatin structure of sperm cells, as well as DNA-binding proteins, and methods used by an oocyte for integration of introduced genes, might lead to lower efficiency of rabbit ICSI-T procedure (Moisyadi et al., 2009; Vasicek et al., 2010).

Another important observation made in the present investigation was the presence of significant concentration-dependency in terms of toxic effect observed in the process of increasing DNA concentration from 10 to 20 ng/µl. Such an effect could be seen using all delivery methods; however, its influence on the number of embryos produced was especially noticeable in the PNI and ICSI-T groups. In particular, there was a 70% reduction in blastocyst formation when using pronuclear injection, and no blastocysts could be formed in the ICSI-T group with a high DNA concentration. Previously, similar observations have been made in other transgenic animals, indicating that the introduction of a large amount of DNA may result in disruption of the embryogenesis process (Chrenek et al., 2005; Garcia, 2018). Consequently, the results from the current study highlight the significance of attaining the optimal DNA concentration, which is an important factor influencing transgenic embryo generation.

It should be noted that the observed toxicity may become especially significant in case when the use of transposition systems like piggyBac is used. As distinct from common plasmid vectors, piggyBac promotes DNA mobility by utilizing a transposase enzyme to cut and paste DNA into the target site for successful chromosomal integration (Song et al., 2020). However, although this characteristic increases transgene insertion frequency, it may also result in higher levels of genomic stress that lead to developmental complications. Thus, a decrease in blastocyst formation rate recorded after reaching 20 ng/µl of DNA concentration demonstrates that it is important to strike a balance between increasing efficiency of transgene insertion and reducing toxicity effects. This problem can also be observed in the course of research conducted while producing transgenic animals.

The current results are also consistent with earlier transgenic experiments in rabbits, which have shown better embryo development with reduced DNA concentration levels. In particular, Chrenek et al. (2005) succeeded in obtaining blastocyst development rates close to 90%, significantly higher than those recorded in the present experiment. Nevertheless, the researchers used a lower amount of DNA and differing embryo culture conditions, which may suggest that the amount of DNA is an important variable influencing the development process. Moreover, rabbit transgenesis via sperm mediated gene transfer has demonstrated varied success based on the methods of DNA preparation and sperm manipulation (Vasicek et al., 2010). All these results seem to indicate that lowering the DNA level below 10 ng/µl might contribute to improved embryo development.

An interesting point was that blastocysts positive for GFP were present in significantly higher numbers in the group of embryos that survived PNI when DNA was added at 20 ng/µl than in the other group where DNA was used at 10 ng/µl. Even though higher amounts of DNA led to a decrease in embryo survival rate, the embryos that survived had higher percentages of transgene expression frequencies. This could be explained by the fact that only embryos that are able to integrate and tolerate the presence of transgenes survive up to blastocyst stage. This kind of selective pressure was noted in other works that described the transgenic production process in animals using conventional microinjection as well as genome editing approaches (Song et al., 2013; Xu et al., 2021).

The findings of this investigation carry significant importance regarding future use of rabbits in medical research. Rabbits are being extensively used to model diseases such as cardiovascular problems, metabolic

diseases, immunodeficiencies, eye diseases, and recombinant proteins due to similarities in physiology compared with rodents used commonly in scientific researches (Wang et al., 2013; Matsuhisa et al., 2020; Xu et al., 2021). Additionally, genetic engineering of rabbits through TALENs and CRISPR/Cas9 methods has opened up new possibilities for the creation of animal models for various diseases (Song et al., 2013; Song et al., 2017; Yuan et al., 2017). However, it should be stressed that the ability to efficiently manipulate embryos as well as introduce exogenous genetic material is an essential requirement before using the aforementioned genome editing tools. The current investigation has determined the optimal technique of transgenesis based on pronuclear microinjection of the piggyBac transposon at 10 ng/µl concentration.

More broadly, the optimization of the rabbit transgenesis procedure is especially pertinent in the context of scientific organizations located in developing/emerging nations. Compared to larger livestock species, rabbits need relatively few resources and expenses associated with their maintenance, but at the same time, they are a good intermediate model between mice and humans (Matsuhisa et al., 2020; Xu et al., 2021). Thus, the developed criteria for measuring blastocyst formation rate and GFP-expression in embryos can be applied by other laboratories working in the field of rabbit transgenesis as an indication point for measuring their success.

In general, the current study suggests that pronuclear microinjection is the best strategy to obtain transgenic embryos if used in combination with an enhanced piggyBac transposon vector system. Moreover, the DNA concentration plays an important role in embryo viability and expression of the transgene, with 10 ng/µl being the optimum concentration in this regard. In conclusion, the obtained results may be considered another step towards optimization of the transgenic rabbit production process and used in subsequent works to assess transgene integration into the genome and germline transmission.

Limitations

In the current experiment, development was only evaluated up to the blastocyst stage in vitro. It is yet to be determined whether the blastocysts created using these techniques will implant and develop into full-term offspring following embryo transfer. Although the formation of blastocysts and the expression of GFP are important steps towards establishing transgenesis, this cannot be proven without having offspring, since transgene integration into the germline can only be verified in offspring. Furthermore, it was not evaluated how many copies of the transgene were transferred into the rabbit embryos and at which location in the genome, meaning that we do not know whether or not the piggyBac system successfully made cut-and-paste transposition and the observed GFP expression is due to transient plasmid expression.

6. Conclusion

The current study evaluated three methods of gene delivery for the production of transgenic rabbit embryos and showed that the use of PNI with 10 ng/µl DNA concentration allows achieving maximal blastocyst rate (42.1% of cleaved embryos) while ensuring acceptable levels of transgene expression (37.5% of blastocysts are GFP positive). The use of ICGI resulted in relatively low blastocyst rates (approximately 10–11%) irrespective of DNA concentration, and only at the lower concentration (66.6% of blastocysts were GFP positive). The use of ISPI transgenesis resulted in absence of GFP-expressing blastocysts; besides, only one blastocyst was obtained during ISPI transgenesis, which shows that this technique is inappropriate for obtaining transgenic rabbit embryos under the conditions of this study. Increased DNA concentration from 10 to 20 ng/µl affected embryo viability negatively in all studied techniques, although this effect was not equally strong for each of them. In case when total amount of transgenic embryos should be prioritized by researchers, PNI with 10 ng/µl DNA concentration should be used.

This study can be further advanced through transferring blastocysts generated using optimized conditions to a recipient female in order to evaluate the rates of term development and germline transmission. Investigation into lower DNA concentrations (1-5 ng/µL) for all three injection techniques will lead to conditions where there is an optimal balance between DNA toxicity and its integration efficiency, unlike those explored in this study. Further investigations focusing on the mechanisms responsible for DNA

localization, rate of degradation, and innate immune response upon introduction of DNA into rabbit zygotes via various methods of delivery can help in explaining the differences in concentration responses among the injection techniques.

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