EFFECTS OF SEPERATE OR SIMULTANEOUS INJECTION OF TWO DIFFERENT GENES (ENHANCED GREEN-FLUORESCENCE PROTEIN GENE, HUMAN GAMMA INTERFERON GENE) ON TRANSGENIC MICE RECOVERY*

İki Farklı Genin (Güçlendirilmiş Yeşil Floresan Protein Geni, İnsan Gamma İnterferon Geni) Ayrı Ayrı veya Birlikte Mikroenjeksiyonunun Transgenik Fare Eldesi Üzerine Etkilerinin Araştırılması*

Korhan ARSLAN[1], Haydar BAĞIŞ[2], Munis DÜNDAR[3]

Summary: *Sunulan çalışmada hIFN-γ ve EGFP gen konstraktlarının farklı konsantrasyonları birlikte ve ayrı ayrı pronüklear safhadaki fare embriyolarına mikroenjekte edilmiştir. Mikroenjeksiyon deneyleri aktarılan gen konstraktlarının yalnız ve/veya birlikte aktarımlarına göre üç farklı deney grubu çalışılmıştır. I.Deney Grubunda hIFN-γ ve EGFP genleri birlikte mikroenjekte edilmiş, aktarımlar sonucunda toplam 39 yavru doğmuştur. Yapılan moleküler analizlerde toplam üç yavrunun transgenik olduğu belirlenmiştir. Üç transgenik yavrudan iki'si EGFP transgenik, bir tanesi ise hIFN-γ transgeniktir. II. Deney Grubunda yalnız hIFN-γ geni mikroenjekte edilmiş, aktarımlar sonucunda toplam 21 yavru doğmuştur. Yapılan moleküler analizlerde toplam iki yavrunun hIFN-γ transgenik olduğu belirlenmiştir. III.Deney Grubunda yalnız EGFP geni mikroenjekte edilmiş, aktarımlar sonucunda toplam beş yavru doğmuştur. Bu yavrulardan elde edilen DNA'lar ile yapılan moleküler analizlerde transgenik yavruya rastlanmamıştır. Sunulan çalışma da elde edilen sonuçlar ile iki farklı genin birlikte mikroenjeksiyonu sonunda doğan yavruların transgenik olma yüzdelerinin yüksek olduğu ortaya konmuştur. Sunulan çalışmada geliştirilen protokolün transgenik fare üretim çalışmalarında uygulanması durumunda; iki farklı geni ayrı ayrı veya birlikte taşıyan transgenik fare hatları elde edilebilecek, böylece ekonomi ve zaman açısından tasarruf sağlanabilecektir.*

Anahtar kelimeler: *Transgen, mikroenjeksiyon, EGFP, hIFN-γ, Ko-enjeksiyon*

Özet: *In this study, different concentrations of hIFN-γ and EGFP gene constructs were separately or simultaneously injected to mouse embryos at pronuclear phase. Micro-injection were classified in three different experiment groups according to alone or simultaneous transfer of gen constructs. In first group co-injection of hIFN-γ+EGFP trans-genes were performed. As a result of transfers, totally 39 offsprings were born, In molecular analysis performed on DNAs obtained from those offsprings, two were EGFP transgenic and one was hIFN-γ transgenic. In second group micro-injection of hIFN-γ trans-gene was performed. As a result of transfers, totally 21 offsprings were born. In molecular analysis performed on DNAs obtained from those offsprings totally two neonates were hIFN-γ transgenic. In third group micro-injection of EGFP trans-gene was performed. As a result of transfers, totally five offsprings were born, it was found that there were no transgenic offspring. Results presented in present study found that transgenicity percentage of offsprings born as a result of simultaneous micro-injection of two different genes was high. If the protocol presented in this study is applied to transgenic mice recovery studies, it is possible to recover transgenic mice carrying two different genes separate. Thus, it will be possible to save money and time.*

Key words: *Transgene, Micro-injection, EGFP,*

[1] *Arş.Gör.Dr.Erciyes Ün.Vet.Fak.Genetik AD, Kayseri*

[2] *Doç.Dr.TÜBİTAK MAM Gen Müh.Enstitüsü, Kocaeli*

[3] *Prof.Dr.Erciyes Ün.Tıp Fak.Tıbbi Genetik AD, Kayseri*

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Animals that bear in their genome a recombinant gene pertaining to another organism are called transgenic animals (1). Studies of the reproduction of transgenic animals aim to contribute to various branches of medicine such as understanding genetic functions, designing disease modelling and pharmacology (2,3,4). In addition, knowledge of intercellular interaction mechanisms in immume system, and of gene regulatory mechanisms also provide valuable contributions to the studies of developmental biology (5,6,7). The most favored method of reproducing transgenic beings is that of pronuclear microinjection (2,4,8). Gene transfer is done using the technique of microinjection into the pronucleus of oocytes (zygots) in their monocellular phase (1). The first study to produce a transgenic animal was realized in 1974 on mice by using the DNA of SV40 virus, a retrovirus (9,10,11). Gordon et al. made significant progress by demonstrating for the first time that the DNA of a foreign organism was integrated into a fertilized mouse embryo using the method of microinjection (3). It has been reported that in the studies performed the foreign DNA has been irretrievably integrated into the mouse genome and these genes are expressed (3,12,13). A successful gene integration along the mouse lines was first realised at the stage of preimplantation by means of Murine Leukemia virus (14).

The transgene (gene construct) to be transferred consists of two main sites. The first of these is the transcriptional unit of the transferred gene, which comprises exons, basically protein encoding areas, and introns, areas not encoding proteins (15,16). The second, however, is the area where regulatory elements are located by which the control is provided of the expression of the gene known with various names such as promoter, enhancer, and reporter (15,16). The first of the two transgenes used in this study is the human gamma interferone (hIFN-γ) controlled by the rat why acidic protein (WAP) promoter. WAP promoter is a powerful one specific to mouse mammary tissue (17). hIFN-γ is a particularly powerful cytokin with immunomodulatory charecteristics (18), and is reported to have been in use in the treatment of a multitude of diseases employing recombinant DNA technology (19). The second is the enhancer green fluorescent protein (EGFP) bound to the transgene cytolomegalovirus (CMV) promotor. CMV is a promoter frequently used in transgenic studies (20). The current study aims to investigate the effects of microinjections, together or individually, and at diffeent cocentrations, of EGFP, the gene responsible for the enforced green fluorescent protein expression, and the gene hIFN-γ, on the reproduction of transgenic mouse.

MATERIAL AND METHOD

Gene constructs microinjected

EGFP transgene to be used in microinjection has been prepared from PEGFP-N1

(BD Bioscience Clontech, Catalogue # 6085-1) plazmit DNAin such a way as to include both the area of promoter CMV, the regulatory elements of the gene together with the gene EGFP, and SV40 poly A signal. pEGFP -N1 plazmit DNA has been transformed into HB101 competent cells and the mixture of the transformed cell and DNA has been diffused on luria-bertoni (LB) agar. The cells have been left to grow overnight. The cells having antibiotic resistance have been sorted out and placed, using sterile pipette tips, individually in their places LB liquid food prepared in separate tubes. The cells have been left to grow overnight at 37º C in an incubator. pEGFP-N1 has been isolated from the feeding sites where growth has occured. The isolated pEGFP-N1 plazmit has been cut off using Afl II and VSP I restriction enzymes .The products cut off has been introduced into agar gel of 1% and subjected to electrophoresis. The gel part with the EGFP transgenic structure of 1632 base pair(bp) has been cut off and eluted from the gel with the elusion kit (Promega, Wizard SV gel and PCR Clean Up System A9281). The eluted pure EGFP transgene DNA has been diluted in varying concentrations in microinjection buffer (10 mM Tris-HCL ph 7.5; 0.15 mM EDTA distilled water added until the total volume reached 10 ml) and prepared for microinjection.

The second transgene in the study, WAP-hIFN-γ sized 5191 bp, was cut using the enzymes *Sal*I and *Eco*RI, and the hIFN-γ transgene of 2383 bp was removed. WAP-hIFN-γ construct has been prepared by the researchers of TÜBİTAK (Turkish Scientific Research Committee) MAM GMBE Transgene and Experimental Animals Laboratory.

Group of Animal Subjects

Used as donor in the study for the procurement of embryos were a total of 79 5-6-week-old female hybrid mice of CB6/F1 reproduced through the mating of C57BL76J x BALD/C. Thirty hybrid male stud mice of CB/F1 were used for mating with the donors. For the synchronization of the receipents, however, 40 vasectomized male mice of CB6/F1 were used. For receipent mothers, 82 10-12-week-old female mice of the CDI stock. Ethic committee report for the animals used in the study was obtained from TÜBİTAK-MAM-GMBE Ethic Committee of Animal Experiments.

Microinjection and Embryo transfer

Microinjection experiments were divided into three different experimental groups depending on the gene construct being transferred alone and/or together. In the first experimental group two different gene constructs (hIFN-γ and EGFB) were microinjected together. In the second experimental group the mictoinjection of the gene construct hIFN-γ only was realized, while the gene construct EGFP alone was microinjected in the third group. The concentration of the constructs was set to be 2-5 ng/µl with the microinjection buffer. The transgene EGFP was diluted with the microinjection buffer at the concentrations of 2 ng/µl, 2.5 ng/µl, and 5 ng/µl; the gene construct hIFN-γ at 2 ng/µl, 4 ng/µl, and 5 ng/µl before injection. Microinjection was realized using a microinjection set comprised of an invert microscope with a magnification of 20 (Zeiss,Axiovert-35 M), ependorf micromanipulators, ependorf grasping pipette (Model 5242)and an automatic microinjector (21). The transfer of the embryos to receipent mothers was performed immediately after microinjection using the method reported by Bağış et al. (22).

Detention of Transgene Presence with PCR

Genomic DNA was obtained from the tail tissue using the method described by Hogan et al.(23). For the detection of the presence of EGFP and hIFN-γ transgene, possible transgenic offsprings were scanned molecularly using PCR method. In the PCR reactions set up for EGFP scan use was made of 100 ng/µl of DNA, primers of 20 pmol/µl, and 2xPCR Master Mix (fermentas). The sequences of the primers that multiplied the specific area of 719 bp are 5'-ATGGTGAGCAAGGGCGAGGAGCTGT-3' (Sense),3'-TACAGCTCGTCCATGCCGAGAGTGATCC-5' (Antisens) . EGFP-NI plazmit DNA of

1 ng/µl was used as positive control. The DNA's went through the protocol in which subsequent to 5-min. preliminary denaturation at 95º C, they were subjected to 30 cycles each consisting of 1 min.-binding at 58,7º C, 1.5 min.-extension at 72ºC followed by the 10 min.-extension protocol at 72º C after the final cycle. In the PCR reactions set up for hIFN-γ scan use was made of 100 ng/µl of DNA, primers of 20 pmol/µl, 2x PCR Master Mix (Fermentas). The sequences of the primers that multiplied the specific area of 496 bp are 5'-CCAGGAGAAGTCACCCTCAGATG-3' (Reverse). WAP-hIFN-γ plazmit DNA of

1 ng/µl was used as positive control. The DNA's went through the protocol in which a 5-min. preliminary denaturation at 95º C, they were subjected to 30 cycles each consisting of 1 min.-binding at 60º C, 1.5 min.-extension at 72º C, followed by the 10 min.-extension protocol at 72º C after the final cycle.

Statistical Analyse

Differences between birth ratio of different concentration of microinjected transgens in different trail groups was analysed by means of chi-square test. And also effects of seperate or simultaneous injection of two different genes in birth ratio was analysed by means of Chi-Square test.

RESULTS

The Effect of Transgenes in Different Concentrations on Transgenic Reproduction

The transgene hIFN-γ+EGFP, which was coinjected in the first experimental group, was prepared in three different concentrations and microinjected. It was found that the number of the young produced as a result of co-microinjection of the transgenes with the concentration of 2 ng/µl was greater than that of the young produced from the microinjection at other concentrations ($p<0.001$). Of the embryos into which the transgenes with the concentration of 2ng/µl were microinjected together, 267 were transferred to the foster mothers. As a result, 28 baby mice were born. The molecular analyses of the DNA's from these young mice revealed that one of them bore the gene hIFN-γ (3,5%). Of the embryos into which the transgenes prepared at the concentration of 4 ng/µl were microinjected together, 202 were transferred to foster_mothers. From this transfer came 4 baby mice. In the molecular analyses of the DNA's from these baby mice were encountered no transgenic offspring. Of the embryos into which the transgenes at the concentration of 5 ng/µl were microinjected together, 290 were transferred to foster mothers, and 7 baby mice were born. The molecular analyses of the DNA's from these baby mice showed that 2 of them born the gene EGFP (28,5%) (Table I). The transgene hIFN-γ used in the second experimental group was prepared in two different concentrations and microinjected. The statistical difference between the numbers of the young reproduced from the microinjection of the gene constructs was shown to be nonsignificant ($p>0.05$). Of the embryos into which the transgene prepared at the concentration of 2 ng/µl was microinjected, 182 were transferred to foster mothers. The molecular analyses of the DNA's from the ten young mice resulting from this transfer demonstrated that one of them was hIFN-γ transgenic (10%). Of the embryos into which the transgene at the concentration of 4 ng/µl was microinjected, 336 were transferred to foster mothers with the result that 11 baby mice were born. It was detected at the molecular analyses of the DNA's that one of these baby mice was hIFN-γ transgenic (9%) (Table II). EGFP transgene used in the third experimental group was prepared in three different concentrations and microinjected. Of the embryos into which the transgene of 2 ng/µl was microinjected, 208 were transferred to foster mothers. Nevertheless, no birth occurred. As for the gene prepared at the concentration of 2.5 ng/µl ,155 of them were microinjected to foster mothers, which resulted in the birth of five baby mice. The DNA's taken from these baby mice were analysed but no transgenic baby was found. Finally, 68 of the embryos microinjected into the transgene at the concentration of 4 ng/µl were transferred but no birth occurred (Table III).

The Ratio of Microinjection and Transgenic

Table I. Differences between birth ratio of different concentration of hIFN-γ+EGFP transgens

hIFN-γ+EGFP transgen concentration (ng/µl)	Number of microinjected embryo	Number of offsprings(%)	EGFP Transgenic Mice	hIFN-γ Transgenic Mice
2	267	28 (%10,4)	-	1 (%3,5)
4	202	4 (%1,98)	-	-
5	290	7 (%2,41)	2 (%28,5)	-

Table II. Differences between birth ratio of different concentration of hIFN-γ transgen

hIFN-γ transgen concentration (ng/µl)	Number of microinjected embryo	Number of offsprings	hIFN-γ Transgenic mice
2	182	10 (%5,49)	1 (%10)
4	336	11 (%3,27)	1 (%9,0)

Table III. Differences between birth ratio of different concentration of EGFP transgen

EGFP transgen concentration (ng/µl)	Number of microinjected embryo	Number of offspring (%)	EGFP Transgenic Mice
2	208	-	-
2,5	155	5 (%3,22)	-
4	68	-	-

Offspring

A total of 16 trials were performed on the first experimental group. In the experiments 759 microinjected embryos were transferred to foster mothers, which resulted in the birth of 39 offsprings. In the molecular analyses of the DNA's taken from these offsprings it was observed that three offsprings were transgenic. Of these, two were EGFP transgenic and one was hIFN-γ transgenic. The total number of the trials in the second experimental group treated with the microinjection of hIFN-γ transgene was 12. In the trials was realized the transference of 518 microinjected embryos to_foster mothers, which resulted in 21 offsprings. The molecular analyses of the DNA's from these offsprings revealed that two of them were hIFN-γ transgenic. In the third experimental group treated with the microinjection of EGFP transgene, the total number of the trials performed was seven. In these trials 431 microinjected embryos were transferred to_foster mothers, which resulted in 5 offsprings. No transgenic offspring was encountered in the molecular analyses of the DNA's from these offsprings (Table IV).

Molecular Demonstration of Transgene Presence

The absence or presence of EGFP transgene was detected through PCR analyses. The scans revealed the presence of product of 719 bp belonging EGFP transgene (Fig.1).

Thus, it was found that these two animals bore EGFP transgene, i.e. they were transgenic. The absence or presence of hIFN-γ transgene was also detected through PCR analyses. From the PCR analyses of possibly transgenic mice , the product of 496 bp belonging to hIFN-γ transgene was detected in the PCR products of three mice (Fig.2, Fig.3, Fig.4).

In other words, these three animals were found to bear hIFN-γ transgene and, therefore, be transgenic.

DISCUSSION

Table IV. Comparision of microinjection results

			Transgenic offsprings		**Total offsprings**
			+	-	+
Transgens	hIFNγ+EGFP	Offsprings	3	36	39
		%	7,7	92,3	100,0
	hIFN-γ	Offsprings	2	19	21
		%	9,5	90,5	100,0
	EGFP	Offsprings	0	5	5
		%	0	100,0	100,0
Total		Offsprings	5	60	65
		%	7,7	92,3	100,0

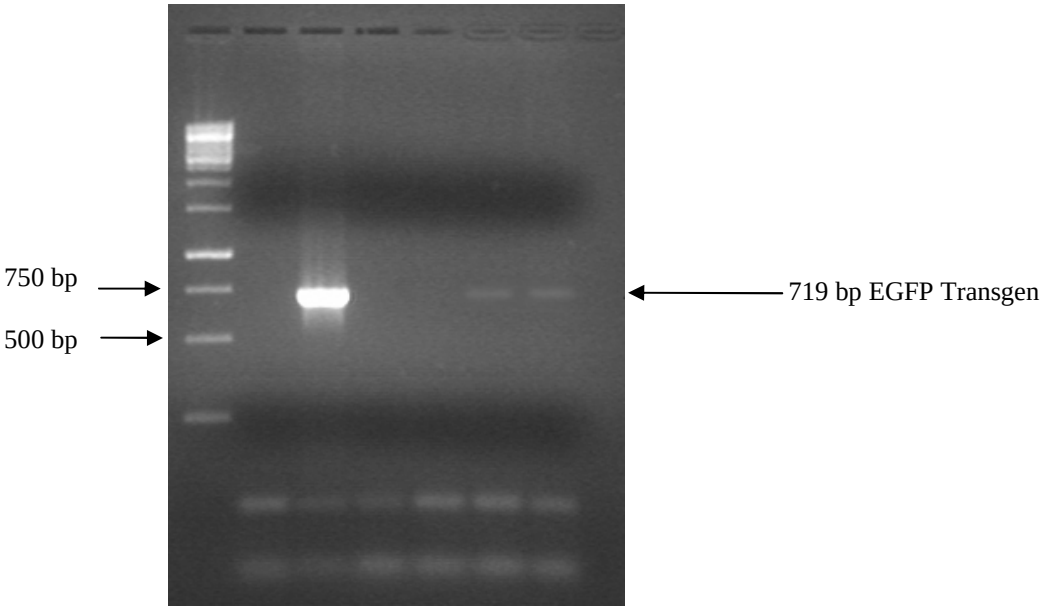

Fig .1 Demonstration of the presence of the EGFP transgene by PCR analysis **M**. 1kb DNA size marker**, 1.** PCR product of nontransgenic mouse as a negative control, **2.** EGFP-N1 plasmid construct as a positive control, **3-4**. PCR products of EGFP transgenic mice.

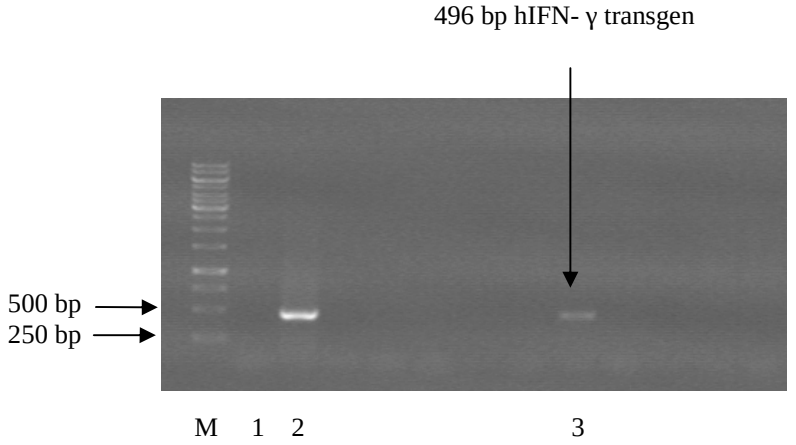

Fig.2 Demonstration of the presence of the hIFN-γ transgene by PCR analysis. **M.** 1kb DNA size marker **1**. PCR product of nontransgenic mouse as a negative control **2**. WAP-hIFN-γ plasmid construct as a positive control, **3**. PCR products of hIFN-γ transgenic mice

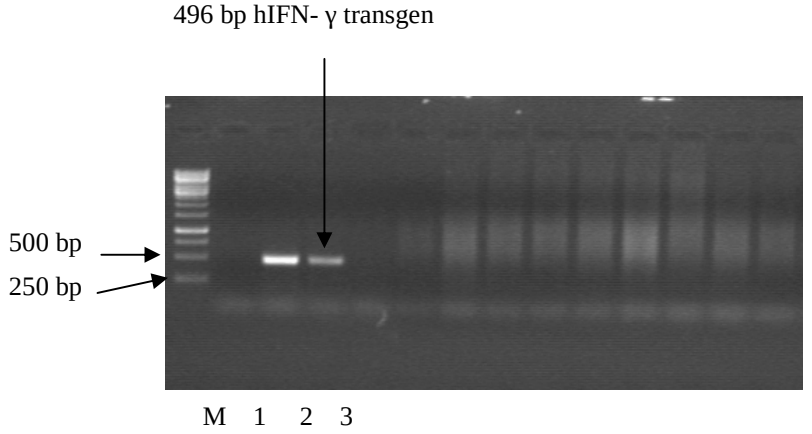

Fig. 3 Demonstration of the presence of the hIFN-γ transgene by PCR analysis **M.** 1kb DNA size marker **1**. PCR product of nontransgenic mouse as a negative control **2**. WAP-hIFN-γ plasmid construct as a positive control, **3**. PCR products of hIFN-γ transgenic mice

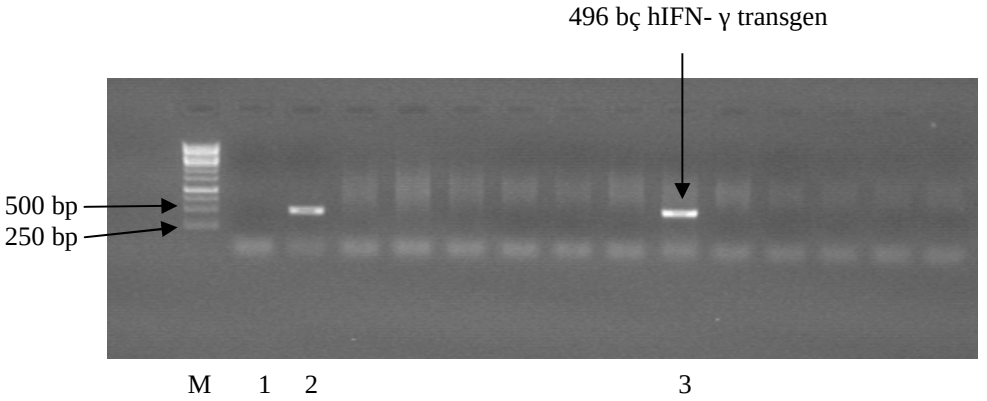

Fig. 4 Demonstration of the presence of the hIFN-γ transgene by PCR analysis **M.** 1kb DNA size marker **1**. PCR product of nontransgenic mouse as a negative control **2**. WAP-hIFN-γ plasmid construct as a positive control,, **3**. PCR products of hIFN-γ transgenic mice

In the study different concentrations of the gene constructs were microinjected together or singly into mice embryos in their pronuclear phase. The ratio of transgeneity in the offsprings obtained was consistent with that found in the study by Zhang et al. (24). In the first experimental group, eproduction of the offsprings bearing two different genes separately is considered to be important for the study presented. Yamamura et al. have reported that in their study in which they have realized the microinjection of a single gene construct the offsprings born have been transgenic by 6.6% (25). It has been reported that in a similar study by Deborah et al. the transgeneity ratio of the young born of pronuclear microinjection was 7%, as detected through the molecular analyses of the young (26). The second experimental group received the microinjection of the gene construct hIFN-γ only. The results of transgenic offspring from this group are in agreement with those in the literature. In the third experimental group the microinjection of EGFP gene construct singly was realized. The molecular analyses of the offsprings born revealed no transgeneity, which raised the probability that there might be a problem to do withe the optimization of the microinjection trials in the third experimental group. However, absence of problem in the first and second experimental group in connection with the reproduction of transgenic young, and the realization of the optimization of the microinjection trials in the third experimental group in the same circumstances as in the other twogroups erased this doubt. That no transgenic offspring was reproduced in the third experimental group raised the hypothesis that the gene constructs and their concentrations employed in the study might have lowered the integration success of the gene EGFP (27). It has been demonstrated through statistical assessments that the microinjection of gene construct being singly or together had no significant effect on the transgeneity of the offsprings obtained ($p>0.05$). Transgene technology has gained great importance in the last two decades and begun to be used in the fields of biomedicine, veterinary medicine, and agriculture. With the application of the advances in the field of genetics to the current transgenic technology and the invention of new technologies will accelerate the reproduction of transgenic animals, which, in turn, will cause a variety of commercial practices. Since transgenic animals will grow faster, be more resistant to diseases, and have effective metabolisms, transgenic technology appears to be promising. The findings from the

study suggest that the percentage of transgeneity in the offsprings born of the microinjection of two different genes together is high. The implementation in the transgenic mice reproduction of the protocol developed in the current study will make it possible to obtain transgenic mice lines that bear two different genes, separately or together, which, in turn, means saving time and money.

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