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Electroporation to Deliver Plasmid DNA into Rat Dental Tissues

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Abstract

Background—Delivery of DNA into the target tissues is an important technique in gene function studies and gene therapy. Surgical treatment of tooth eruption disorders, such as impacted third molars, is a major health care cost. Because the dental follicle (DF) is essential for regulating tooth eruption, establishment of local gene transfer protocols is needed to determine the effect of various genes on eruption and to develop gene therapy approaches to induce the eruption of impacted molars.

Methods—Plasmids containing *lacZ* reporter gene were injected into rat mandibles and then electroporated at the designated settings. Mandibles were collected 24 hours after electroporation for X-gal staining to evaluate the transfection efficiency. Tissues were collected at various days post-electroporation to determine the expression of the transgene.

Results—For the DF, depth of injection and pulse number appear to be important. Six pulses can achieve above 80% transfection of the DF at 50V or 120V. For AB transfection, voltages are important with 120V being optimal. Regarding pulse durations, we determined that durations of 20 ms and 30 ms achieve the maximum transfection in AB and DF, respectively.

Conclusions—This study demonstrates for the first time the feasibility of electroporation to locally deliver plasmids into dental tissues. Parameters affecting electroporation to deliver plasmids into the dental tissues were optimized. This protocol could be used to deliver shRNA or genes of interest into the dental tissues to regulate tooth eruption. Thus, it may be possible to develop non-surgical treatments for inducing eruption of impacted teeth.

Keywords

Electroporation; Transfection; Dental Follicle (DF); Alveolar Bone (AB); Tooth Eruption

Introduction

The dental follicle (DF), a loose connective tissue sac that surrounds the unerupted tooth, is required for tooth eruption. This was first shown in surgical studies whereby when the DF was removed, the tooth did not erupt (1). However, if the DF was left intact and an inert object substituted for the tooth, the inert object erupted (2). At the cellular and molecular level, the DF acts to regulate the osteoclastogenesis and osteogenesis needed for eruption (3,4). Regarding osteoclastogenesis, in the rat first mandibular molar there is a maximal burst of osteoclastogenesis at day 3 postnatally which is brought about in part by upregulation of colony-stimulating growth factor (5) which in turn down-regulates osteoprotegerin (OPG) expression to allow osteoclastogenesis to occur (6).

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No conflicts of interest exist.

Tooth eruption also requires alveolar bone growth in the base of the tooth crypt to serve as an eruption force (7). Rapid bone growth starts at postnatal day 9 in the base of tooth crypt of the rat first mandibular molar. We have proposed that the DF up-regulates the bone growth by increasing expression of osteogenic genes, such as BMP2, which is expressed in the basal one half of the DF (8). To determine if osteogenic genes are indeed causal for the alveolar bone growth needed for eruption, a novel *in vivo* approach is required. Use of null mutations for many of the osteogenic genes, including BMP-2, is lethal, thereby eliminating that approach. Delivery to the DF of selected osteogenic genes to over-express a given gene or delivery of a siRNA targeted against a given osteogenic gene should elucidate the role of each gene in eruption, however. Because tooth eruption is a localized event (3,4), delivery of the gene or siRNA directly to the DF will reduce the uncertainties induced by systemic injection of either the siRNA or the gene product.

Delivery of a plasmid vector into the dental follicle and its adjacent tissues *in vivo* has not been reported. Thus, establishment of a protocol for *in vivo* transfection of DF is needed for elucidating functions of candidate genes for tooth eruption, as well as for developing non-surgical gene therapy treatment for eruption disorders. Three types of techniques (biological, chemical and physical methods) have been successfully used for gene transfer. Of these gene transfer techniques, electroporation, a physical method, is simple, inexpensive and relatively safe. Electroporation gene transfer is based on increasing the permeability of the cell membrane by electric pulsing to allow DNA to enter the cells. Electroporation and electrophoresis are reported to be involved in electrotransfer of plasmid DNA into cells (9,10,11). Such an approach has been successfully used to transfer genes to both soft and hard tissues; e.g., brain (12) and cartilage (13). Furthermore, this method enables one to transfer DNA to specific regions or tissues (14) and thus meets the criteria for transferring genes to the DF and alveolar bone (AB) to affect the localized event of tooth eruption.

We previously have shown that electroporation can be used to efficiently deliver plasmid DNA into dental follicle cells *in vitro*. In this report, we show that *in vivo* delivery of plasmid DNA into the rat intact DF and AB can be achieved by optimizing electroporation parameters. Thus, it should be possible to inject siRNAs targeted against various osteogenic genes to determine which genes are required for the alveolar bone formation seen during eruption. Of special interest is the possibility of delivering specific genes to the DF to promote eruption of impacted teeth, be it human third molars or instances of primary failure of eruption.

Materials and Methods

Plasmid Vector

The vector used in this study is a circular (supercoiled) plasmid containing a bacterial *lacZ* reporter gene driven by a CMV promoter with the total size of 6558 bp. The plasmid was isolated from the host cells (*E. coli*) using Invitrogen Maxi prep kit (Invitrogen Corp, Carlsbad, CA, USA) for transfection experiments. This plasmid has been previously used to transfect cultured dental follicle cells (15). In this study, the plasmid was dissolved in TE buffer and diluted to 1 µg/ul with saline. For electroporation, 20 µl (i.e., 20 µg or 4.7 pmoles) of the plasmid solution was injected into each side (labial and mesial) of the 1st mandibular molar of the rat pups.

In vivo Electroporation

Harlan Sprague-Dawley rats were housed in the School of Veterinary Medicine at Louisiana State University with the protocol approved by the Institutional Animal Care and Use Committee. For electroporation experiments, newborn pups at postnatal days 6 to 8 were

anesthetized with isoflurane and the plasmid was injected into the mandible using a 31G insulin syringe with a stopper attached to the needle to control depth of injection as shown in Fig. 1A. Self-manufactured forceps electrodes were connected to a BTX ECM850 Electro Square Porator (Harvard Apparatus, Holliston, MA, USA), which can generate square-wave pulses. The forceps electrodes were clamped to the given tooth mandible as schematically shown in Fig. 1. The gap between electrodes was about 2.5 ± 0.2 mm, varying according to the size of the mandibles. The electroporation was conducted with 4 pulses at 75V (300 V/cm) for 20 ms per pulse in 1sec intervals, unless otherwise specified. After electroporation, the pups were returned to the cage with their mother. The pups were sacrificed and mandibles were collected for analysis of transfections 24 hours after electroporation.

Histological Analysis of Transgene Expression

For analysis of transfection, frozen rat mandibles were embedded in 10 % carboxymethyl cellulose and cut into 20 μ m longitudinal sections at -25°C with a Leica CM3050 S cryostat. The sections were placed on lysine coated slides, and LacZ expression was detected by X-gal staining. The slides were fixed with 2 % formaldehyde and 0.2 % glutaraldehyde in PBS buffer for 5 min, rinsed with PBS, and incubated in X-gal staining buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 10 % DMSO and 1 mg/ml X-gal in a humidity chamber at 37°C for 4–5 hours, which was previously optimized such that only tissues expressing *lacZ* transgene stained. The slides then were rinsed with PBS and stained with eosin.

RT-PCR analysis of Transgene Expression

To determine the longevity of the transgene expression, AB and first mandibular molars were sampled and collected into TRI reagent (Molecular Research Center, Cincinnati, OH) daily from day 2 to day 6 post-electroporation. Total RNA was extracted from the samples. Following the DNase digestion to remove possible DNA contamination, RNA was quantitated with a Nanodrop spectrophotometer. The RNA then was reverse transcribed into cDNA using random primers. Next, conventional PCR was conducted by mixing 2 μ l of cDNA, PCR buffer, Taq DNA polymerase with *LacZ* or β -actin specific primers respectively. The PCR was carried out with 32 cycles at 94°C for 45 s, 55°C for 1 min and 72°C for 1 min. In parallel, RNA samples were directly used instead of cDNA in PCR reactions to ensure the amplification signals were generated from the RNA. The PCR product was electrophoresed in an agarose gel containing ethidium bromide, and the gel was examined under UV light.

Experimental Data and Statistical Analysis

After X-gal staining, the sections were examined for blue staining to determine the transfection. Tissues with any blue staining were considered as transfected, and the transfection levels were evaluated on a scale of 1 to 5 with 5 being the best. Specifically, levels 1 to 5 denoted the following: transfection occurred in a given tissue less than 5 % (level 1), 5 % to 20 % (level 2), 21 % to 40 % (level 3), 41 % to 60 % (level 4) and greater than 60 % (level 5). Tissues without blue stain indicated no transfection, and were scored as 0. The percentage of transfection was calculated by dividing the number of transfected samples by the total number of electroporated samples (N).

For statistical analysis, the rank-sum test was conducted to obtain the mean rank-sum scores, which reflect the combinations of the frequency and levels of transfection. A higher rank-sum score indicates higher overall transfection level. Both mean rank-sum scores and percentage of transfection were compared to evaluate the treatment effects.

Results

In vivo Transfection

Transfection was detected by X-gal staining. Blue staining indicated that the plasmid DNA was successfully delivered and expressed in the tissues. Tissues (AB or DF) with visible blue staining were classified as transfected samples that were used for calculation of the transfection rate (i.e., % transfection = transfected samples/total samples $\times$ 100). The transfected samples were further scored into 5 levels (scores) according to their degrees of transfection with level 1 being the least and 5 being the strongest transfection. Tissues with no visible blue staining were classified as non-transfected samples and scored as 0. These scores were used for rank-sum tests to statistically compare the transfection.

Different patterns of blue staining were observed in the mandibles that were subjected to plasmid injection and electroporation (Fig. 2A–D), whereas controls without plasmid injection and electroporation did not stain (Fig. 2I). Spatially, transfection could be in the basal part of the given molar (Fig. 2A, B); in the coronal part of the molar (Fig. 2C) or in the entire molar (Fig. 2D). Transfection could occur only in a particular tissue such as in the DF (Fig. 2E) or in the AB (Fig. 2F). Transfection was often seen in several tissues; e.g., DF, stellate reticulum and AB (Fig. 2G). Mandibles receiving plasmid injection but no electroporation did not show any blue staining (Fig. 2H), suggesting that the plasmid DNA cannot spontaneously enter the DF and AB cells.

Effect of the Electrode Polarity on Transfection

The electroporation was conducted by clamping the mandibular molar crypt with forceps electrodes. To determine if the electric current directions affect transfection, the anode was either placed on the labial or on the lingual side of the crypt with the cathode on the opposite side. When the anode was placed on the labial side, 45 % of the electroporated crypts showed some transfection in the DF (Fig. 3A). In contrast, 36 % of the crypts had transfected DFs (Fig. 3A) when the anode was placed on lingual side. For AB, the transfection rates were 81 % vs. 72 % when the anode was placed on the labial vs. lingual side respectively. Thus, it appeared that a better transfection was achieved when the anode was placed on the labial side. However, rank-sum test indicated that the difference was not statistically significant for both transfection of the DF and AB.

Depth of Plasmid Injection

The target transfection site in this study was the DF or its adjacent tissues. To determine the importance of the injection site and to optimize the depth of the injection to reach the target tissues, plasmids were injected into the molar area at depths of 1 mm and 1.8 mm beneath the surface. Electroporation was conducted immediately after the injection with 4 pulses at 75V, 20 ms. For transfection of AB, the mean rank-sum scores were 26.1 in 1 mm-depth injection and 25.9 in 1.8 mm-depth injection, and the difference was not statistically significant. However, for transfection of the DF, a 1 mm-depth injection resulted in a mean rank-sum score of 20.6 whereas 1.8 mm depth injection resulted in the mean rank-sum score of 28.9. The difference was statistically significant ($P=0.019$) (Fig. 3B).

Voltage, Pulse Number and Pulse Duration

Experiments were conducted to optimize the pulsing voltages and the number of pulses on transfection with a 3 (voltage) $\times$ 3 (pulse number) factorial design. Data were analyzed to compare the effect of each treatment (voltage-pulsing number), as well as the main effect of voltage and pulse number (Table 1). For transfection of the DF, the maximal transfection, in terms of transfection rate (85.7 %) and rank-sum scores was achieved at treatment of 120V(480V/cm)- 6 pulses, which is significantly higher than the treatment of 50V (200 V/

cm) - 2 pulses. However, comparable transfection rate (80.0 %) also was seen in the treatment of 50V- 6 pulses (Table 1A). For AB, transfection rate of 100 % was achieved in the treatments of 75V (300 V/cm) - 6 pulses, 120V- 2 pulses or 120V-4 pulses. Maximum rank-sum score was seen at 120V-6 pulses, indicating this treatment resulted in higher level of transfection (Table 1A). Regarding the main effect of voltage and pulse number on transfection, higher voltage or more pulses generally resulted in better transfection than lower voltage or fewer pulses as determined by transfection rate and rank-sum scores (Table 1B, C).

We tested pulse durations of 10 to 40 ms using relative low voltage of 75V (300 V/cm) at 4 square-wave pulses. Optimal pulse durations for transfection of AB and DF were 20 ms and 30 ms, respectively. Pulse duration of 20 ms resulted in a significant higher mean rank-sum score than 30 and 40 ms for transfection of AB, but no significant difference was seen between 10 and 20 ms. For transfection of the DF, increased transfection was seen in pulse durations up to 30 ms. However, no significant difference between treatments was detected with rank-sum test for transfection of the DF (Fig. 3C).

Plasmid Diffusion and Transfection

To determine if the transfection could be improved if the plasmids were allowed to diffuse in the tissue, electroporation was conducted at different times (0–30 min) after plasmid injection. As seen in Fig. 4, the staining was concentrated in the dental area when electroporation was conducted immediately or 5 min after injection (Fig. 4A, B). Staining was faded and scattered in other areas when the electroporation was conducted 10 min or 30 min after plasmid injection (Fig. 4C, D).

To determine the longevity of the gene expression, tissue samples were collected from the transfected pups daily up to 6 days after electroporation. *LacZ* transgene expression was seen in all days except day 6 in alveolar bone, although variation of expression level was observed in different days (Fig. 5). High level of transgene transcripts (mRNA) could still be seen 6 days post-electroporation in the molars (Fig. 5). Amplification of *LacZ* signal was only seen in the RT-PCR, but not in the parallel PCR using RNA as the template (data not shown), indicating the amplification was truly from the transcription (i.e., mRNA) of the *LacZ* transgene.

Discussion

The results of this study clearly demonstrate the feasibility of *in vivo* delivery of plasmid DNA into the DF and AB by electroporation. The electric current likely generates an electrophoresis effect to help accumulation of plasmid molecules at the cell membrane (9), and subsequently, the plasmids cross the membranes that have become transiently permeable by electric pulses (10,16). Thus, the transfection is confined to the local area that received electric pulses (Fig 2). In turn, this local transfection could be useful for development of a gene therapy protocol for treating eruption disorders because tooth eruption is a localized event. Electroporation-based gene therapies have demonstrated the effectiveness of the technique with plasmids. Currently, several phase I and II clinical trials using electroporation-based gene therapy have been conducted for treatment of various diseases (17) However, no similar study has been conducted for treatment of tooth eruption disorders, possibly because of lack of reliable methods for delivery of DNA into dental tissues. Hence, the protocol developed in this study is particularly valuable.

Plasmid DNA is a negatively charged molecule, and thus it moves toward the anode (+) in an electric field. Study of tumor tissue electroporation suggests that plasmid DNA migration speed can be affected by pulsing strength and extracellular matrix composition, such as

collagen content (18). Theoretically, if the plasmid concentration is equally dispersed within the tissue, and if the structure of cell membranes is homogenous, the electric current directions should have no effect on electroporation. However, in this study, a slightly higher transfection was seen when the anode was placed on the labial side rather than the lingual side (Fig. 3A). Because the plasmids were injected into the lingual side of the mandible and because they are negatively charged, the subsequent electroporation with the anode (+) on the labial side would result in their movement toward the labial side such that plasmids are spread and achieve an overall higher transfection. The result also implies that the electrophoresis effect during electroporation cannot be neglected because the electric field may cause the migration of the plasmids within the tissue (11).

Injection depth appeared to have more effect on transfection of the DF than the AB (Fig 3B). Such results would be expected given that the DF is ensconced in the bony crypt and thus a deeper injection would be required to place the plasmids in the DF as compared to the AB. This result also indicates that precise injection into the target tissue is critical to achieve desired tissue transfection with electroporation.

The plasmids can diffuse in the tissue after injection. The diffusion speed is negatively related to the plasmid size; i.e., large plasmids diffuse slower than small plasmids (19). In this study, however, we show that reduced transfection occurred if the electroporation was delayed even 10 minutes after the plasmid injection (Fig. 4), suggesting that electroporation should be conducted as soon as possible or no later than 5 min (Fig. 4A, B). The possible reasons for reduced transfection if electroporation is delayed could be that the plasmids in the tissues may rapidly diffuse out of the tissues before electroporation is done, or that the plasmids were degraded by nucleases if they were not immediately electroporated into the cells. Other studies have shown that naked plasmid DNA can be rapidly degraded by nucleases in the blood after *in vivo* injection (20).

The parameters that modulate the efficacy of transfection after injection and electroporation have been addressed in this study. Electric field strength is an important parameter and several studies suggest that the optimal field strength varies for different tissues (21). In this study, transfection of the DF is maximal at 120 V (480 V/cm)- 6 pulses; however, a lower voltage also can achieve a comparable result (Table 1). For transfection of the AB, voltages greater than 75 V (300 V/cm) are needed to reach the maximal transfection (Table 1). The mineralized matrix of bone may be why a higher voltage is required in comparison to the soft tissue of the DF.

Pulses of low voltage and long duration have been found to increase DNA uptake in tissues *in vivo* (22,23). In a muscle transfection experiment, increasing transfection efficiency was seen with durations up to 40 ms at 200 V/cm, 8 pulses of electroporation (23). In this study, for transfection of AB, the optimal duration was around 20 ms at 75 V (300V/cm), 4 pulses (Fig. 3C). For transfection of the DF, the transfection efficiency continued to increase up to 30 ms, suggesting that transfection of soft tissues may require a longer duration than transfection of hard tissues (e.g., bone).

Because of its small size, transfection of the rat DF is difficult and 100% transfection rate is not always achieved. However, the target tissue of expression of osteogenic genes in the DF is the AB, and the AB is the other tissue that is primarily transfected in addition to the DF. Transfection of AB seems easier than transfection of DF (Table 1), likely because of its relatively large size. Thus, in future studies to transfect osteogenic genes or inhibitors of such genes into the DF, the spillover into the alveolar bone may still result in an effect on bone formation, and, in turn, affect tooth eruption.

The longevity of the transgene expression in the transfected tissues is of importance in designing gene therapy experiments. For example, to study the effect of a given gene on bone formation needed for eruption, the transgene ideally should express at the critical time of osteogenesis in eruption. In that vein, rapid bone growth needed for eruption of the first mandibular molar of the rat is seen at postnatal day 9 until the time of eruption around day 18 (7). Thus, an effective time to start gene transfer for modification of the bone formation needed in tooth eruption should be prior to postnatal day 9, such as days 6 to 8. Given that we have shown that the expression of the transgene can last at least 6 days post-transfection in the dental tissues, transfection of the appropriate gene(s) around day 9 likely should affect bone formation over the critical time period. Although a second transfection could be done at day 15, other electroporation studies to transfect muscle tissue with plasmids did not show a significant reduction of transgene expression until 10 days post-electroporation (24). In addition, long-term expression of a transgene has been reported, in which stable expression was seen in muscle for as long as 9 months post-electroporation (23). In embryonic mouse brain and in rat skin, the transgene can maintain expression for more than 6 weeks or 7 weeks post-electroporation, respectively (25,26). Regardless, these studies demonstrate longevity of transgene expression in the tissues *in vivo* such that transfection would not have to be done daily.

Conclusion

The DF regulates tooth eruption through its regulation of alveolar bone formation and resorption that are needed for tooth eruption (3,4). This study shows that plasmids can be delivered to the dental follicle (DF) and surrounding alveolar bone (AB). Higher electric field strength appears to favor transfection of hard tissue, such as AB, whereas a longer pulse duration may be necessary for optimal transfection of soft tissue such as the DF. The transgene can remain stably expressed in the dental tissues *in vivo* for at least 6 days after transfection.

Use of the plasmid containing the *lacZ* reporter gene facilitates the examination of the transfection efficiency. The *lacZ* gene can be replaced by the genes of interest in gene function and gene therapy studies. Alternatively, other plasmid vectors containing genes of interest can be delivered to the DF and the AB by electroporation. When plasmid vectors containing genes for bone formation or resorption are delivered into such dental tissues, they are expected to modify the eruption.

Because tooth eruption is a localized event, the local transfection of the dental tissues seen in this study suggests that injection and electroporation might be used to affect the eruption of a single tooth. The results of this study are significant in aiding the development of human gene therapy for treating eruption disorders. The DF and the AB of humans are much larger than that of the animals (rat pups) used in this study. Consequently, an increase in the voltage, pulsing duration and pulsing number may be necessary. However, precise injection of the vector into the DF and the AB is expected to be much easier in humans because of the larger tissue size.

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Fig. 1. Plasmids were injected into the mandibles of rat postnatal pups with a syringe attached by a stopper to control the injection depth (A). Instruments used for electroporation in this study included a self-generated forceps electrodes connected to a BTX ECM850 Electro Square Porator. The diagram shows the electrodes clamped on the labial and lingual sides of the tooth crypt for electroporation (B).

Fig. 2. Transfection of rat dental follicle and its adjacent tissues by electroporation with *lacZ* plasmid. The blue staining indicates that the *lacZ* gene was delivered and expressed in the dental tissues. Panels A–D are low power micrographs showing different patterns of transfection as seen in the basal part of the molar (A, B); in the coronal part of the molar (C); and around the entire molar (D). Panels E–G are higher power micrographs showing the detailed transfection as seen in the dental follicle (E); in the alveolar bone (F); and in the dental follicle and other dental tissues such as stellate reticulum (G). No staining is seen either in the mandible injected with plasmid, but without electroporation (H), or in the negative control without plasmid injection and without electroporation (I).

Fig. 3. Electroporation to transfect *lacZ* plasmid into rat dental tissues. (A) Effect of electrode polarity on % transfection of alveolar bone (AB) and dental follicle (DF). Total sample size=23; (B) Rank-sum test to compare the effect of plasmid injection depth on transfection of AB and DF. Note that a significant difference was seen between injection depths of 1.0 and 1.8 mm for transfection of DF as indicated by **. Total sample size= 51; (C) Rank-sum test to compare the effect of pulse durations on transfection of AB and DF. Note that 20 ms duration resulted in the maximum transfection of AB which is significantly higher than durations 30 and 40 ms as indicated by different letters. No significant difference duration effect was detected for transfection of DF. Total sample size=24.

Fig. 4.

Effect of plasmid diffusion on transfection of the dental tissues. The plasmids were allowed to diffuse at the times indicated after injection prior to electroporation. Note that the blue staining (arrows) was seen around the molar when the mandible was electroporated immediately (0 min) or 5 min after injection (A, B), but less staining (arrow) was seen when electroporation was conducted 10 or 30 min after injection (C, D).

Fig. 5.

Expression of *LacZ* transgene in alveolar bone and the first molar of rat pups after electroporation to transfect plasmids, as determined by RT-PCR and electrophoresis in agarose gel. Tissue samples were collected at the days indicated post-electroporation. Amplification was seen for both bone and molar samples at all days except day 6 in bone. No amplification was seen in the negative control without cDNA in the PCR. The positive controls using *LacZ* plasmid and dental follicle cell cDNA indicate the correct amplification of the *LacZ* and β -actin genes in the PCR reactions.

Table 1

Optimization of electroporation to transfect the dental follicle and alveolar bone

(A) Effect of voltage and pulsing number in a 3×3 factorial design									
Treatment		Sample (N)	Transfection of Dental Follicle			Transfection of Alveolar Bone			Rank-sum score*
Voltage (V)	Pulse #		#	%	Rank-sum score*	#	%	Rank-sum score*	
50	2	14	6	42.9	47.3A	11	78.6	53.5A	
50	4	14	10	71.4	57.6AB	12	85.7	55.2A	
50	6	10	8	80.0	68.4AB	8	80.0	46.2AB	
75	2	14	10	71.4	61.0AB	12	85.7	51.7AB	
75	4	14	9	64.3	60.1AB	11	78.5	69.0AB	
75	6	14	9	64.3	64.8AB	14	100	68.1AB	
120	2	14	8	57.1	57.0AB	14	100	71.1AB	
120	4	14	10	71.4	63.3AB	14	100	68.1AB	
120	6	14	12	85.7	75.9BC	13	92.8	82.0BC	

(B) Effects of voltage irrespective of pulse number									
Voltage	Sample (N)	Transfection of Dental Follicle			Transfection of Alveolar Bone			Rank-sum score*	
		#	%	Rank-sum score*	#	%	Rank-sum score*		
50V	38	24	63.2	56.7A	31	81.5	54.4A		
75V	42	28	66.7	62.0A	37	88.1	55.7A		
120V	42	30	71.4	65.4B	41	97.6	73.7A		

(C) Effects of pulse number irrespective of voltage amount									
Pulse #	Sample (N)	Transfection of Dental Follicle			Transfection of Alveolar Bone			Rank-sum score*	
		#	%	Rank-sum score*	#	%	Rank-sum score*		
2	42	24	57.1	55.1A	38	90.5	58.8A		
4	42	29	69.0	60.3A	37	88.1	56.3A		
6	38	29	78.1	69.8A	35	92.1	70.2A		

*The mean rank-sums scores with a same letter are not significantly different.