Journal of Oral and Maxillofacial Surgery, Medicine, and Pathology 24 (2012) 49–53

Contents lists available at SciVerse ScienceDirect

Journal of Oral and Maxillofacial Surgery, Medicine, and Pathology

journal homepage: www.elsevier.com/locate/jomsmp

Original research

BMP-2 gene transfer under various conditions with *in vivo* electroporation and bone induction

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ARTICLE INFO

Article history:
Received 26 February 2010
Received in revised form 9 May 2011
Accepted 13 September 2011

Keywords:
BMP
Gene transfer
In vivo electroporation
Low voltage
Bone regeneration

ABSTRACT

In our previous study, we successfully induced bone formation in rat skeletal muscle using a gene-transfer system, in which a non-viral BMP-2 expression vector was applied to the muscle by *in vivo* electroporation at 100 V. With the ultimate goal of applying this method to maxillofacial bone regeneration therapy, we sought to establish a safer system in which the gene is transferred into the target area with a lower voltage, but with the same efficiency. The *LacZ* or *BMP-2* gene was transferred using *in vivo* electroporation under various conditions: 25–100 V, 50–200-ms loading time, and 8–128 pulses. The gene-transfer efficiency or bone induction was quantified by measuring β-galactosidase or alkaline phosphatase (ALP) activity and calcium content. Histological, immunohistochemical, and X-ray analyses were also used to examine the effect of the gene transfer. When the voltage for *in vivo* electroporation was lowered, the gene transfer efficiency was also reduced. However, by increasing the loading time or number of pulses, it was possible to improve the gene-transfer efficiency to the same level attained at 100 V, and *in vivo* electroporation under the lower voltage conditions successfully induced new bone. We have established a safe and efficient gene-transfer system for bone regeneration therapy using a non-viral BMP gene expression vector and *in vivo* electroporation.

1. Introduction

Therapies for the safe, targeted delivery of bone morphogenetic protein (BMP) [1,2], a biologically active substance that induces bone formation, are anticipated for clinical use. We have focused on the method of delivery of the BMP gene into a target area. Gene therapy generally uses a viral vector, such as adenovirus [3,4], which can be toxic or induce an immune response [5,6]. Non-viral vectors are safer, but the gene-transfer efficiency of non-viral plasmid vectors is lower than that of viral vectors [7]. For our gene-delivery system, we constructed a non-viral plasmid vector that expresses BMP-2 (pCAGGS-BMP-2) and used *in vivo* electroporation to promote its transfer into rat skeletal muscle [8,9], where ectopic bone formation is easy to evaluate. *In vivo* electroporation is an attractive method because it has high gene-transfer efficiency and is easy, safe, and inexpensive, requiring only a plasmid and a simple device [10–13].

The gene-transfer efficiency of *in vivo* electroporation is affected by various factors, such as the electric voltage, loading time, number of pulses, and type of electrode [11,14,15]. Reported *in vivo* electroporation techniques use standardized parameters, either low voltage and long duration, or high voltage and short duration [11,16]. Although efficient gene transfer is possible with an electric potential of 100 V (low voltage and long duration), we sought to improve the safety of our *in vivo* electroporation method for actual clinical use.

Our final goal is to apply our gene transfer system to bone regeneration therapy in oral and maxillofacial regions. When we applied our system to the rat intraoral region at a level of 100 V, the pulse shock to the target area was relatively strong. Although no electrical injury was observed on the oral mucosa of the rats, each

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doi:10.1016/j.ajoms.2011.10.006

pulse shock at this voltage caused strong head shaking. In clinical practice using electrical stimulation to head region, for psychiatric disorders, some patients lose consciousness when they are treated by electroconvulsive therapy (ECT) [17] at a level of 100 V without general anesthesia. Today, to prevent on muscle convulsion, modified electroconvulsive therapy (m-ECT) with muscle relaxant under general management by anesthesiologists is performed [18]. Although it is successful to take away muscle convulsion, some complications still remained as memory impairments [19]. We concluded that it was too difficult to apply our *in vivo* electropolation system at a level of 100 V for oral and maxillofacial regions like ECT or m-ECT. We need more simple and safe therapy without anesthesia and muscle relaxant for future trials of intraoral bone regeneration.

We reasoned that an appropriate lower voltage would be similar to that of a transcutaneous electrical neural stimulator such as the myomonitor, which is a medical instrument used to treat temporomandibular joint disorder [20], and which uses pulses under 50 V without any anesthesia. In this study, we therefore optimized the parameters for *in vivo* electroporation using voltages below 100 V to achieve efficient gene transfer and bone regeneration.

2. Materials and methods

2.1. Preparation of BMP-2 and LacZ expression vectors

The pCAGGS-BMP-2 and pCAGGS-LacZ expression vectors were described previously [9,10], and were prepared using a Qiagen EndoFree Plasmid Giga Kit (Qiagen GmbH). The plasmids were grown in *Escherichia coli*, DH5α.

2.2. Gene transfer by transcutaneous in vivo electroporation

Nine-week-old male Wister rats (each treatment group $N = 6$) were anesthetized by an intraperitoneal injection of pentobarbital sodium (5.0 mg/100 g of body weight), and the fur on the target area of the leg was removed with clippers. Plate electrodes were coated with keratin cream (Fukuda Denshi) and attached to the skin at the target site transcutaneously. In some cases the skin at the center of the target area was incised with a #11 scalpel, and then held back with forceps so electrodes could be attached directly to the gastrocnemius muscle. The accuracy of the applied electric current was confirmed by measuring the resistance between the electrodes, which were placed opposite one another at the middle of the gastrocnemius muscle. A total of 25 μg plasmid DNA was injected with a 28-gauge needle into the center of the muscle between the electrodes. Electroporation was performed immediately after the injection, using an electroporator. The treatment was initially applied using 8 electrical pulses at 100 V for 50 ms. After these conditions were set as the control, the voltage was reduced to 50 or 25 V with various loading times (50–200 ms), and numbers of pulses (8–128 pulses).

2.3. Measurement of β-galactosidase production

Twenty-four hours after the transfer of the LacZ expression vector, 0.1 g of the gene-transferred muscle was removed and homogenized at 12,000 rpm/min with 5000 μl of the Beta-Glo® Reagent from the Beta Glo Assay System (Promega). This substrate is cleaved by β-galactosidase to form luciferin and galactose. The luciferin was then processed in a firefly luciferase reaction to generate light. After centrifugation, the supernatant was collected and 100 μl was transferred into individual wells of a 96-well plate, in duplicate. Absorbance (OD 420 nm) was measured with a luminometer, and the Relative Light Units (RLU) for each electroporation configuration was determined as the value compared to that at 100 V, 50 ms, and 8 pulses, which was defined as the standard.

2.4. Histological analysis

Twenty-four hours after transfer of the LacZ expression vector, the gene-transferred region was isolated and fixed with 4% paraformaldehyde. Samples were embedded in paraffin wax, cut into 5-μm serial sections, and stained with Hematoxylin-eosin (H-E). For the BMP gene-transfer groups ($N = 1$ for each treatment group), the target region was excised 20 days after the gene transfer, embedded in paraffin wax, sectioned, and stained with H-E.

2.5. Immunohistochemical analysis

The above-described *LacZ*-gene-transfected samples were deparaffinized, rehydrated, and treated with 3% H_2O_2 to block endogenous peroxidase activity. The sections were then incubated with an anti-β-galactosidase antibody (1:400) (Promega) and an HRP-labeled anti-mouse antibody. The labeling reaction was visualized with diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. In the group receiving *BMP-2* gene transfer, muscle sections were taken 10 days after the transfer and were stained with an anti-human BMP-2 antibody (1:400) (Sigma) and an HRP-labeled anti-mouse antibody. Immunohistochemical analysis was performed using the same procedure as for the *LacZ*-gene-transfected samples.

2.6. X-ray analysis

Twenty days after gene transfer by *in vivo* electroporation, the gene-transferred region was surgically removed and analyzed by soft X-ray imaging.

2.7. Biochemical analysis

After soft X-rays were taken, the same samples ($N = 5$) were homogenized at 12,000 rpm/min. After centrifugation, the supernatant was collected and the alkaline phosphatase activity (IU/mg protein) was measured using the 4-nitrophenylphosphate method. In addition, the calcium content of the rest of the sample, except the

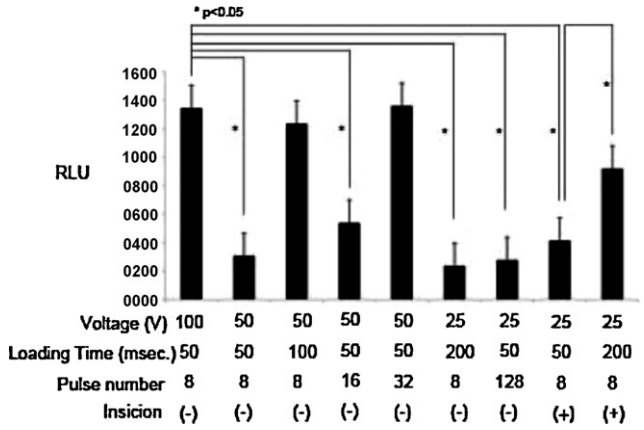


Fig. 1. Gene-transfer efficiency at 50 V and 25 V β-galactosidase production was determined using a substrate that is cleaved by β-galactosidase to generate galactose and luciferin, which was and measured by a luminometer. The Relative Light Unit (RLU) was the value compared to that obtained at 100 V, 50 ms, and 8 pulses, which was defined as the gene-transfer efficiency under standard conditions. The gene-transfer efficiency using 50 V or 25 V and various loading times or pulse numbers is shown. The gene-transfer efficiency when electrodes were placed in direct contact with the target tissue after skin incision is also shown. Each group $N = 5$, significance level 0.05, $^*p < 0.05$, error bars show the standard error of the mean.

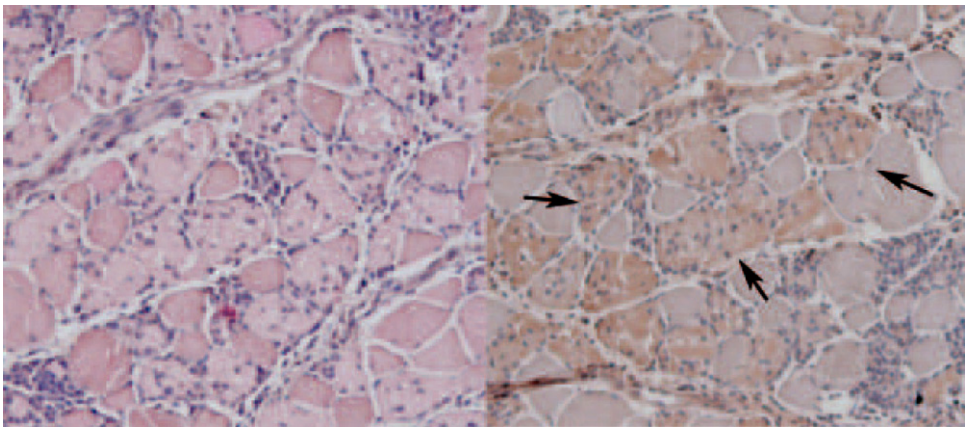


Fig. 2. Histological changes after gene transfer. Histology and immunohistochemical staining using an anti-β-galactosidase antibody 24 h after gene transfer under the conditions of 50 V, 50 ms, and 32 pulses are shown. Arrows point to the β-galactosidase-positive area. 400× magnification.

supernatant, was measured by the orthocresol-phthalein complex method.

2.8. Statistic analyses

Results are presented as the mean ± standard error of the mean (SEM). Statistical analysis of differences in the alkaline phosphatase activity and calcium content between groups was performed by analysis of variance (ANOVA), followed by Fisher's comparison test.

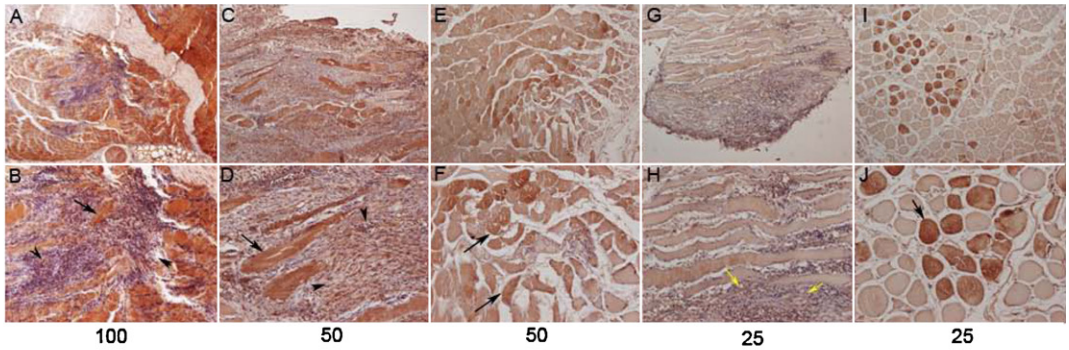
3. Results

3.1. Gene-transfer efficiency under low-voltage conditions

We examined the gene-transfer efficiency by measuring the β-galactosidase production at a *LacZ*-gene-transferred region. The standard conditions were set at 100 V, 50 ms, and 8 pulses. When the voltage was reduced from 100 V to 50 V, the gene-transfer efficiency was significantly reduced (Fig. 1). To increase the gene-transfer efficiency at 50 V to the same level attained at 100 V, 50 ms, the loading time had to be increased to twice the standard time (Fig. 1). Increasing the number of pulses to 16 or 32 with the standard loading time also accelerated the gene-transfer efficiency. In particular, with 32 pulses, the same gene-transfer efficiency was obtained as with the standard conditions (Fig. 1). On the other hand, when the voltage was reduced to 25 V and the loading time was increased to 200 ms with 8 pulses, the gene-transfer efficiency did not recover to the standard level (Fig. 1). When the pulse number was increased to 32 or 128 at 25 V and 50 ms, the efficiency remained quite low (Fig. 1 and data not shown). By performing a skin incision and allowing the electrode to make direct contact with the target region, the gene-transfer efficiency with 25 V and 8 pulses was improved when the loading time was increased from 50 to 200 ms (Fig. 1).

3.2. Effects on the target tissue under low-voltage conditions

None of the animals subjected to gene transfer at 25–100 V exhibited any necrotic areas in the target tissue 24 h after the procedure (Fig. 2; the data for 25 V and 100 V are not shown). To examine the *LacZ*-gene-transferred area in tissue sections, we performed immunohistochemical staining using an anti-β-galactosidase antibody. Many β-galactosidase-positive cells were observed in the same areas populated by inflammatory cells, in the 50 V-treated samples (50 ms, 32 pulses) (Fig. 2 arrows).



Voltage (V)	100	50	50	25	25
Loading Time (msec.)	50	100	50	200	50
Pulse Number	8	8	32	8	128

Fig. 3. BMP-2 production in muscles after gene transfer under low-voltage conditions. Immunohistochemistry for BMP-2 10 days after gene transfer under 100 V, 50 ms, and 8 pulses (A and B), 50 V, 100 ms, 8 pulses (C and D), 50 V, 50 ms, 32 pulses (E and F), 25 V, 200 ms, 8 pulses (G and H), or 25 V, 50 ms, 128 pulses (I and J). A, B, E, F, I, and J are horizontal sections of muscles, and C, D, G, and H are longitudinal sections. Arrows indicate BMP-2-positive cells, arrowheads show BMP-2-positive and hematoxylin-stained cells. Yellow arrows are BMP-2-negative hematoxylin-stained cells. A, C, E, G, and I are 40× magnification; B, D, F, H, and J are 100× magnification.

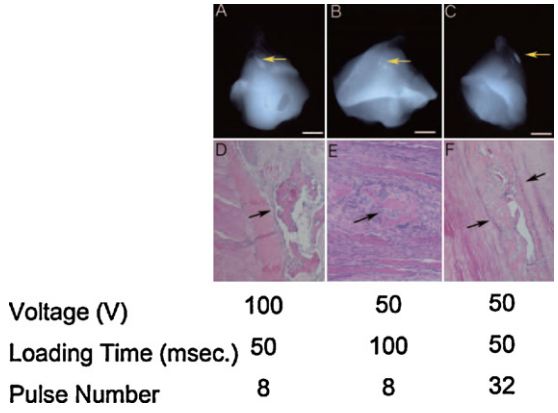


Fig. 4. Bone induction following gene transfer under low-voltage conditions. Bone induction under low-voltage conditions observed by X-ray (A–C) or histological analysis (D–F) is shown. Arrows point to an X-ray-impermeable area (A–C). Histological images of the same area are shown (D–F). The tissue sections in D–F were prepared 20 days after gene transfer. 100× magnification.

3.3. BMP-2 production under various configurations of in vivo electroporation

Muscles stimulated by 100 V, 50 ms, and 8 pulses, 50 V, 100 ms, and 8 pulses, or 50 V, 50 ms, and 32 pulses were strongly positive for BMP-2, and many cells stained with hematoxylin were also positive for BMP-2 (Fig. 3A–F). We observed many spindle-shaped cells (Fig. 3 black arrowheads) as well as muscle tissue with positive staining for BMP-2 (Fig. 3 black arrows) in these sections. Compared to these sections, fewer cells stained positive for BMP-2 in the sections of muscles stimulated with 25 V, 200 ms, and 8 pulses or 25 V, 50 ms, and 128 pulses (Fig. 3G–J black arrows). Although we observed hematoxylin-positive stained cells in longitudinal sections from muscles stimulated with 25 V, 200 ms, and 8 pulses, there were fewer BMP-2 positive cells than in those stimulated with 50 V or 100 V (Fig. 3G and H yellow arrows).

3.4. Bone induction under low-voltage conditions

On the basis of our results using the low-voltage conditions, we transferred the human *BMP-2* gene at 100 V, 50 ms, and 8 pulses (standard conditions); 50 V, 100 ms, and 8 pulses; or 50 V, 50 ms, and 32 pulses. Twenty days later, X-ray impermeable images with an obvious border were observed in all three of these groups (Fig. 4A–C). Histological examination of the tissues subjected to gene transfer from all the groups revealed that the X-ray-impermeable region represented new bone tissue populated by many osteoblasts, osteocytes, and osteoclasts (Fig. 4D–F). Furthermore, 20 days after gene transfer, no significant difference in alkaline phosphatase or calcium content was observed between the tissues treated under the standard conditions of 100 V, 50 ms, and 8 pulses and the low-voltage conditions of 50 V, 50 ms, and 32 pulses. However, both values were significantly higher ($p < 0.05$) at 50 V, 50 ms, and 32 pulses than at 50 V, 100 ms, and 8 pulses (Fig. 5).

4. Discussion

Here, we examined whether we could achieve efficient gene transfer under safer conditions, to establish a rat model of bone regeneration treatment. The gene-transfer efficiency is affected by the electric voltage, loading time, and pulse number used for *in vivo* electroporation [10–12]. Several studies have optimized electroporation conditions to improve the DNA electrotransfer efficacy. These conditions can be classified into two types: high voltage (HV) and short loading time, or low voltage (LV) and long loading time. HV conditions range from 600 V to 1000 V with a 100-μs loading time, and LV conditions range from 80 V to 100 V with a 100–400-ms loading time [21–23].

We previously performed gene transfer for bone induction in the rat intraoral region using *in vivo* electroporation at a low voltage and long loading time, 100 V with a 50-ms loading time and 8 pulses. However, the pulse shock to the target area was undesirably strong. There were no indications of electrical burn, but every pulse shock caused strong head shakes. Indeed, ECT in the treatment of psychiatric disorders is performed at a level of 100 V electrical stimulation by electrodes on the skin of scalp. But, there are several complications included memory impairments and strong muscle convulsion [17,18]. Even though m-ECT can suppress muscle convulsion with muscle relaxant, some complications still remained [19]. Moreover, m-ECT did not spread widely, because it was needed the collaboration with anesthesiologist [17]. We therefore determined that 100 V was too dangerous for clinical use and sought to establish new conditions using an even lower voltage.

When only the voltage was changed, to 50 V and 25 V, the gene-transfer efficiency was also reduced. In this study, by increasing the loading time or pulse number, we were able to reach a similar gene-transfer efficiency with 50 V as with 100 V. With 25 V, however, we were not able to reach the same gene-transfer efficiency as with

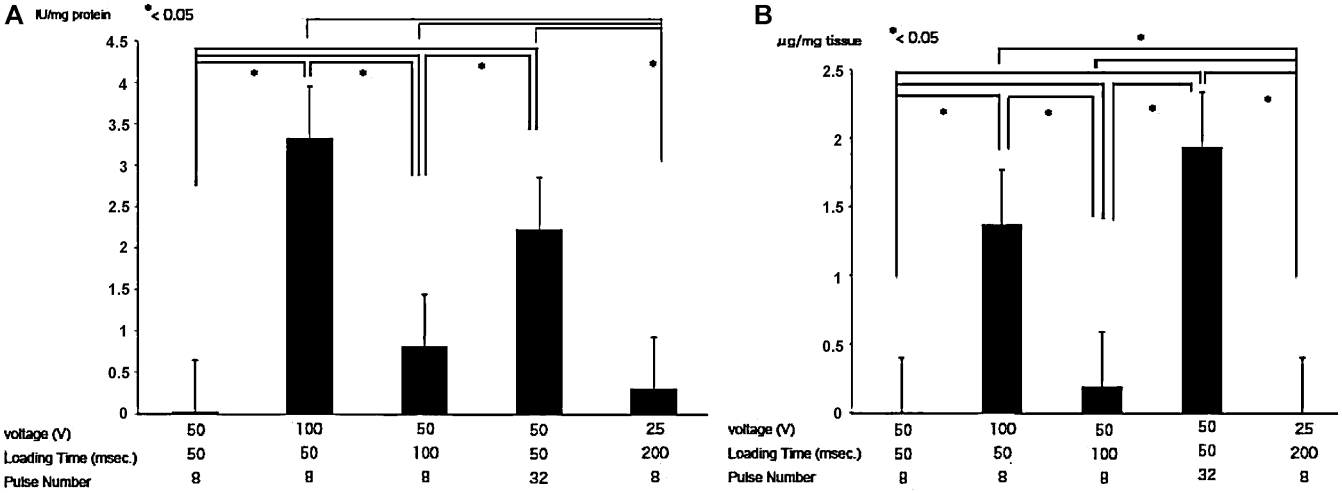


Fig. 5. Alkaline phosphatase activity and calcium content of new bones. The alkaline phosphatase activity (A) and calcium content (B) of new bone induced by BMP gene transfer under low voltage is shown. Each group $N = 5$, significance level 0.05, $^*p < 0.05$, error bars show the standard error of the mean.

100 V by increasing the loading time or pulse number, although the transfer efficiency was slightly improved at 25 V with a prolonged loading time combined with skin incision and direct application of the electrodes to the target muscle.

Consistent with these data, when we examined the results of human *BMP-2* gene transfer under low-voltage conditions, as much bone formation was observed at 50 V as at 100 V. The properties of the new bone were quite similar in the animals treated with the standard versus the low-voltage conditions, although the alkaline phosphatase activity and calcium content were more greatly affected by changes in the pulse number than in the loading time at 50 V. However, as we expected from the results of the *LacZ* gene transfer efficiency at 25 V, we could not find any bone formation in areas treated with 25 V. The immunohistochemical results at 25 V revealed that there were many inflammatory cells stained with hematoxylin, but fewer BMP-2-positive stained cells than were found at 50 or 100 V. We felt that increasing the loading time to as much as 200 ms, or the pulse number up to 128 times, would be more likely to cause tissue damage and would not improve the BMP-2 production in the target area.

In this study, we transferred the *BMP-2* gene into muscle in rats, since it is easy to evaluate the bone formation derived from exogenous BMP-2 production in this location. Our final goal, however, is to apply our *BMP-2* gene transfer process to bone regeneration in the maxillofacial regions. On the basis of these findings, we are planning to perform *BMP-2* gene transfer to subperiosteal areas around the alveolar bone in rats, using *in vivo* electroporation at a level of 50 V. In the future, we expect to develop a new alveolar bone regeneration therapy using this *BMP-2* gene transfer system with *in vivo* electroporation.

Acknowledgements

This study was supported in part by a Grant-in-Aid from the Ryobi Teien Memory Foundation and Industry Promotion Foundation in Okayama.

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