

Communication

***bmp-2* Gene-Transferred Skeletal Muscles with Needle-Type Electrodes as Efficient and Reliable Biomaterials for Bone Regeneration**

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Abstract: Background: Bone morphogenetic protein-2 (*bmp-2*) has a high potential to induce bone tissue formation in skeletal muscles. We developed a bone induction system in skeletal muscles using the *bmp-2* gene through in vivo electroporation. Natural bone tissues with skeletal muscles can be considered potential candidates for biomaterials. However, our previous system using plate-type electrodes did not achieve a 100% success rate in inducing bone tissues in skeletal muscles. In this study, we aimed to enhance the efficiency of bone tissue formation in skeletal muscles by using a non-viral *bmp-2* gene expression plasmid vector (pCAGGS-*bmp-2*) and needle-type electrodes. Methods: We injected the *bmp-2* gene with pCAGGS-*bmp-2* into the skeletal muscles of rats' legs and immediately placed needle-type electrodes there. Skeletal tissues were then observed on the 21st day after gene transfer using soft X-ray and histological analyses. Results: The use of needle-type electrodes resulted in a 100% success rate in inducing bone tissues in skeletal muscles. In contrast, the plate-type electrodes only exhibited a 33% success rate. Thus, needle-type electrodes can be more efficient and reliable for transferring the *bmp-2* gene to skeletal muscles, making them potential biomaterials for repairing bone defects.

Keywords: muscle; bone; *bmp-2*

1. Introduction

Recombinant human bone morphogenetic protein (rhBMP)-2 has a high potential for osteoinduction [1]. In previous studies and clinical applications, absorbable and biocompatible carrier components have been used as biomaterials to retain rhBMP-2 at the target site to achieve successful osteoinductive treatments [1–8]. The rhBMP-2 concentration required for effective osteoinduction ranges from 0.75 to 1.5 mg/mL in clinical trials [9,10]. Long-term high-dose rhBMP-2 treatment leads to osteoclastogenesis via a negative feedback mechanism [11]. Therefore, we developed a gene therapy using a viral or non-viral *bmp-2*

gene expression plasmid vector for bone regeneration [12–14]. However, adenoviral vectors induce an immune response against transduced cells [15,16]. The expression of the human *bmp-2* gene using a viral vector induces bone tissue formation, which occurs only when immune responses are either locally or generally suppressed [15,16]. In our next strategy for safer *bmp-2* gene therapy for bone regeneration, we constructed a non-viral *bmp-2* gene expression plasmid vector. Although non-viral plasmid vectors are safer than viral vectors, their transfection efficiency is usually lower [17–19]. Consequently, strategies are needed to enhance the efficiency of gene transfer to cells using non-viral vectors. Non-viral approaches for gene delivery to cells can be broadly categorized into physical penetration methods, such as electroporation, gene gun, and laser techniques, as well as chemical carriers, including lipofection, lipoplexes, exosomes, synthetic nanoparticles, etc. [20–22]. Moreover, combinations of these methods have been developed, such as photothermal nanomaterial-mediated photoporation [23]. In our first trial, we attempted to increase the transfection efficiency of the gene using a combination of a non-viral *bmp-2* gene expression plasmid vector and in vivo electroporation [12]. We successfully induced ectopic bone formation in skeletal muscles using plate-type electrodes but did not achieve a 100% success rate [12].

It is known that several parameters affect the efficiency of gene transfer by electroporation [24–26]. Electric field distribution, which is one of the parameters, is related to the electrode configuration, as well as electric pulse parameters [27,28]. In the case of gene transfer to the skeletal muscles of rats, electric field distribution is visualized, such as needle-type (Figure 1C) or plate-type (Figure 1D) electrodes. Nonetheless, the electric field distribution with needle-type electrodes is less than that with plate-type electrodes; it cannot be obstructed by the skin, mucosa, and muscle fascia. This is attributed to the ability of needle-type electrodes to reach deep into target areas easily [29,30].

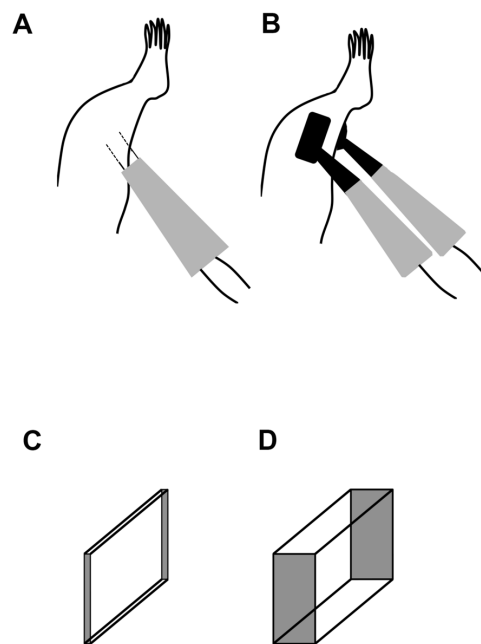


Figure 1. Schematic of the in vivo electroporation setup. (A) Needle-type electrodes were inserted directly into the targeted muscles (dotted line). (B) Plate-type electrodes were attached to the skin of the targeted muscles. The electrical ranges of (C) needle- and (D) plate-type electrodes are two- and three-dimensional, respectively.

Bone graft with muscle could be expected to be a very promising surgery, especially intractable bone fractures, such as for femoral neck fractures, which are notorious for complications like avascular necrosis and nonunion [31,32]. In previous reports, *bmp-2* gene-transferred skeletal muscles with an adenoviral plasmid vector were used to repair

large segmental bone defects in rats [33,34]. *bmp-2* gene-activated muscle grafts with adenoviral vectors exhibit bone volume and stability similar to bone isografts, mimicking autologous bone grafting in rats [34]. Compared with bone autografts, muscle tissues can be harvested in larger quantities, causing only low donor site morbidity [34–38]. Once gene transfer to muscle grafts has been optimized, gene-transferred skeletal muscles may become a more potent material for grafting, with higher osteoinductivity and the ability to promote faster and more robust bone defect healing. Therefore, safe and reliable *bmp-2* gene-transferred skeletal muscles could be used as biomaterials for repairing bone defects.

In this study, we aimed to enhance the efficiency of bone tissue formation in skeletal muscles by using a non-viral *bmp-2* gene expression plasmid vector and needle-type electrodes.

2. Materials and Methods

2.1. Preparation of *bmp-2*-Expression Plasmid

The pCAGGS-*bmp-2* plasmid expressing the human *bmp-2* gene was constructed as previously described [12]. pCAGGS-*bmp-2* contains the CAG (cytomegalovirus immediate-early enhancer/chicken β -actin hybrid) and human *bmp-2* cDNA. The plasmid was transformed into *Escherichia coli* (DH5 α) and isolated using a Qiagen EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany). The DNA was diluted in phosphate-buffered saline (PBS) to a concentration of 0.5 μ g/ μ L before injection into the rat's skeletal muscles.

2.2. Gene Transfer by Electroporation with Needle-Type Electrodes

Nine-week-old male Wistar rats ($n = 6$ for each treatment group) were anesthetized via intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) and the fur on the target leg area was removed with clippers. The plasmid vector was diluted with PBS to a concentration of 0.5 μ g/ μ L and 25 μ L was injected into the middle portion of each gastrocnemius muscle using a syringe with a 31-gauge needle. Needle-type electrodes made of stainless steel (Ohta Seiko Co., Okayama, Japan) with a length of 10 mm, separated by 5 mm, were inserted into the areas previously injected with the plasmid vector (Figure 1A). The surface area of the plate-type electrodes made of stainless steel (Ohta Seiko Co., Okayama, Japan) was 10 mm $\times$ 15 mm (Figure 1B). Electroporation was immediately performed using eight electrical pulses at 100 V for 50 ms with an electroporator (Ohta Seiko Co., Okayama, Japan). All animal experiments were approved by the Animal Research Control Committee of Osaka Dental University (approval no. 19-02016).

2.3. Radiographic Analysis

The rats were sacrificed with an overdose of sodium pentobarbital 21 days after *bmp-2* gene transfer. The injected regions of the gastrocnemius muscles were dissected and calcified tissues were detected on soft X-ray films using SRO-M50 (Sofron Inc., Tokyo, Japan). These images were captured under specific imaging conditions, with a secondary voltage of 28 kV, a current of 2.5 mA, and an exposure time of 30 s.

2.4. Image Analysis

We identified radiopaque areas that contained particles using soft X-ray films, plotted the perimeter of each particle-containing opacity, and measured each area using ImageJ software (access date 15 October 2021) [37]. Measurements were performed five times, and the mean values were calculated.

2.5. Histological Analysis

The rats were euthanized with an overdose of sodium pentobarbital 1 day or 21 days after *bmp-2* gene transfer. The dissected gastrocnemius muscles were fixed with 0.05 M phosphate-buffered 4% paraformaldehyde (pH 7.4) and embedded in paraffin. The skeletal muscles with plate-type electrodes were decalcified, cut into 7 μ m thick sections, and stained with hematoxylin and eosin (HE). The skeletal muscles with needle-type electrodes were cut without decalcification and stained with HE.

2.6. Statistical Analyses

All of the data are presented as mean $\pm$ standard deviation. Statistical significance between the two groups was determined using an unpaired *t*-test. If a *p*-value from the *t*-test, conducted at a 95% confidence interval, was 5% or less, it was considered indicative of a significant difference between the two groups. Multiple group comparisons were performed using one-way ANOVA and Tukey's multiple comparison post hoc tests.

3. Results

3.1. Radiographic Findings

Radiographs revealed opacities comprising several small calcified particles with clear margins in the muscles targeted for *bmp-2* transfection via in vivo electroporation using needle-type electrodes (Figure 2A). The opacities were detected in all six rats (100%). In vivo electroporation using plate-type electrodes resulted in large opacities with clear margins in the targeted muscles (Figure 2B) in two out of six rats (33%). The average areas of each particle-containing opacity were $0.071 \pm 0.048 \text{ mm}^2$ (needle-type electrode) and $0.494 \pm 0.54 \text{ mm}^2$ (plate-type electrode) (Figure 3A). The average areas of total particle-containing opacities were $0.31 \pm 0.376 \text{ mm}^2$ (needle-type electrode) and $1.135 \pm 1.082 \text{ mm}^2$ (plate-type electrode) (Figure 3B).

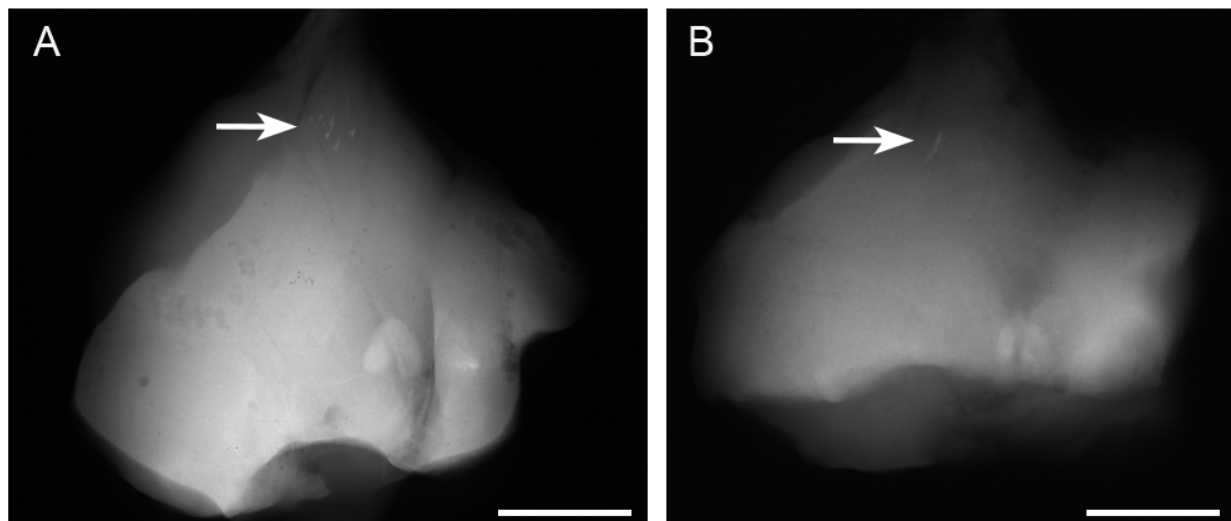


Figure 2. Presence of opacities on soft X-ray films after *bmp-2*-expressing plasmid electroporation. The opacities (white arrow) in the targeted muscles after electroporation with (A) needle- and (B) plate-type electrodes. Several opacities (white arrow) in the skeletal muscle *bmp-2* gene were transferred with needle-type electrodes (A). One opacity (white arrow) was found in the skeletal muscles with plate-type electrodes (B). Each scale bar is 15 mm.

3.2. Histological Findings

To verify whether the opacities detected on soft X-ray films represented ectopic bone formation, we examined muscle sections harvested 21 days after electroporation using HE staining. Several small new bone calcifications were observed in the muscles targeted with the needle-type electrodes (Figure 4A,B, arrows). The ectopic bone tissues (Figure 4B, arrows) were stained slightly lighter with eosin than the muscle tissues (Figure 4B, arrow head) and contained cells resembling osteocytes. These small bone regions were interspersed, similar to the opacities observed on soft X-ray films (Figure 2A). In contrast, the formation of bone calcifications was larger in muscles targeted with plate-type electrodes than in those induced using needle-type electrodes. Moreover, the muscles targeted with plate-type electrodes contained bone-marrow-like tissues, including lipid-rich tissues and hematocytes (Figure 4D, arrow). Muscles targeted with empty plasmids did not exhibit ectopic bone formation (Figure 4E,F).

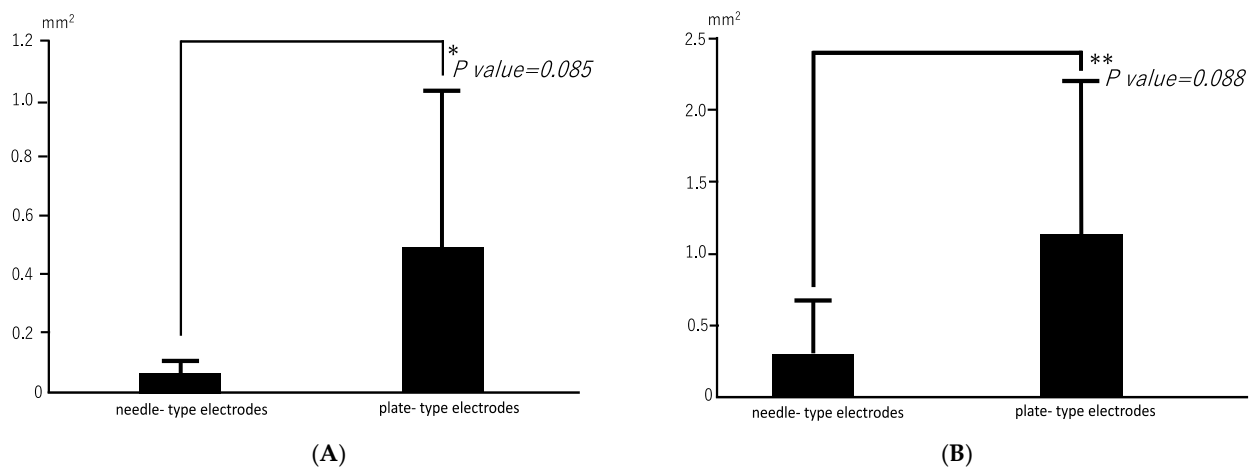


Figure 3. Mean areas of opacities in electroporated skeletal muscles. (A) Mean area for each opacity. * $p = 0.085$; 95% confidence interval: 0.2556–0.1063. (B) Mean total area for opacities. ** $p = 0.088$; 95% confidence interval: 0.750–0.2279.

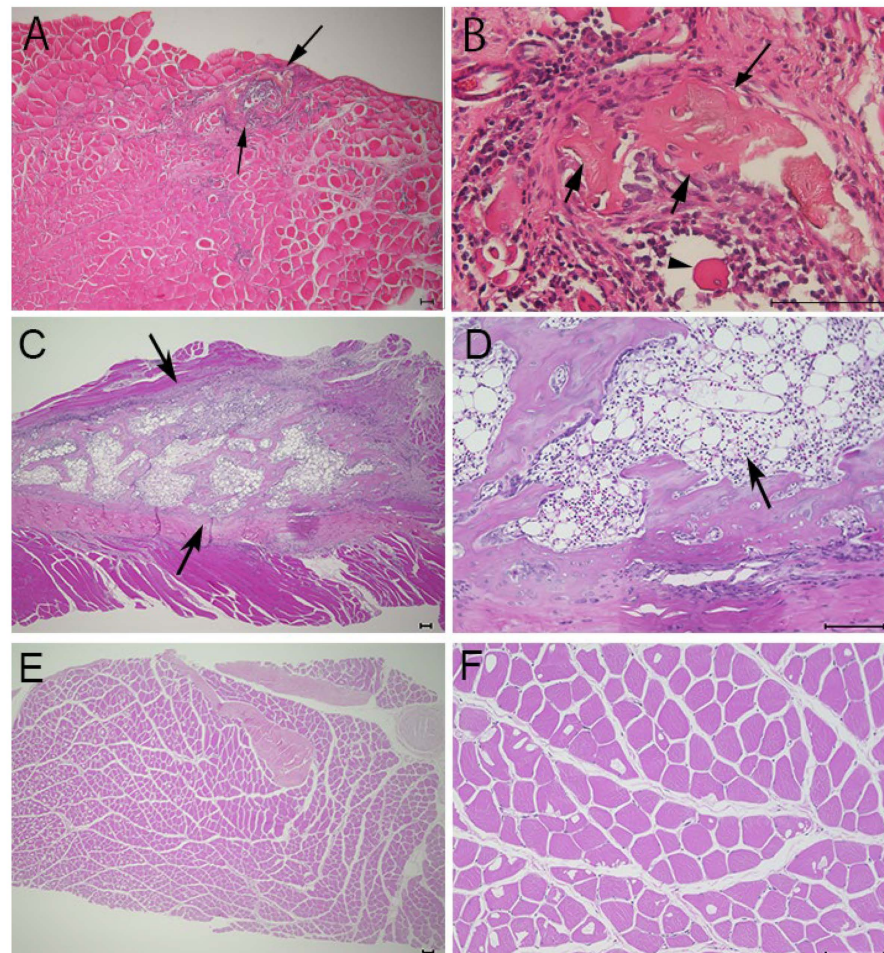


Figure 4. HE staining of targeted skeletal muscles 21 days after gene transfer. HE staining of muscles after electroporation with needle-type electrodes revealed the formation of several bone regions ((A,B), arrows). HE staining of muscles after electroporation with plate-type electrodes showed the formation of larger bone regions ((C), arrows) and lipid-rich tissues and hematocytes ((D), arrow). (E,F) HE staining of the muscles after electroporation with empty plasmid did not reveal the formation of any bone regions. Scale bars represent 100 µm.

We also examined muscle sections harvested 1 day after electroporation using HE staining (Figure 5A,B). The area of muscle fiber damage caused by gene transfer by needle-type electrodes (Figure 5A, arrow head) was smaller than that caused by plate-type electrodes (Figure 5B, arrow head). Inflammatory cells strongly stained with HE were more widespread in areas electroporated using needle-type electrodes than in those electroporated using plate-type electrodes (Figure 5A,B, arrows).

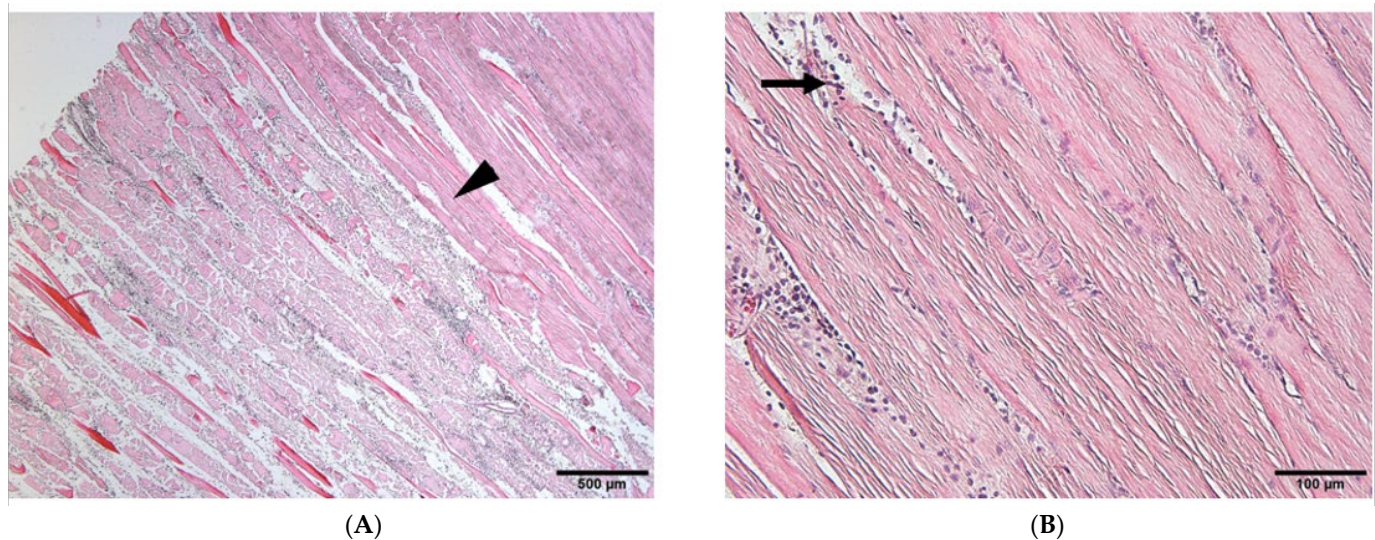


Figure 5. HE staining of the muscles 1 day after *bmp-2* transfer by needle- or plate-type electrodes. The damage area after *bmp-2* transfer by in vivo electroporation with plate-type electrodes (B) was wider than that with needle-type electrodes (arrow head). Inflammatory cells strongly stained with HE were more widespread in areas electroporated using needle-type electrodes than in those electroporated using plate-type electrodes (arrow) (A).

4. Discussion

We successfully induced bone tissue formation in the targeted muscles using needle-type electrodes with a 100% success rate. In contrast, plate-type electrodes achieved only a 33% rate of inducing bone tissues. Our previous study revealed that a low voltage (<25 V) could not efficiently transfer a non-viral plasmid vector using plate-type electrodes without a skin incision, unlike the efficiency achieved with 100 V. However, when we performed a skin incision and directly applied plate-type electrodes on the muscles, we efficiently transferred a non-viral plasmid vector [39]. In this study, we performed gene transfer using plate-type electrodes without skin incision to reduce invasion to the target area. In contrast, needle-type electrodes were directly inserted into the targeted muscles, allowing for easier and more efficient tuning of electricity, which is known to affect the efficiency of gene transfer [39–41]. Although we need to consider other factors, such as the biocompatibility of electrode materials [42], needle-type electrodes may be suitable for gene transfer into skeletal muscles.

However, *bmp-4* gene-transferred muscles did not achieve a 100% success rate for osteoinduction with needle-type electrodes [43]. This may be derived from different types of BMP family members and different plasmid vectors. A comprehensive analysis of the osteogenic activity of 14 types of BMPs in osteoblastic progenitor cells suggested an osteogenic hierarchical model in which *bmp-2*, -6, and -9, not *bmp-4*, may play an important role in inducing the osteoblast differentiation of mesenchymal stem cells [44]. Moreover, a rat mandibular bone regeneration model required between 1 and 10 µg of *bmp-2* protein administration, whereas more than 10 µg of *bmp-4* protein administration was needed [45]. The pCAGGS plasmid vector has a high potential for gene expression in comparison with pMiw II, which is used for *bmp-4* gene expression [43] because it contains the cytomegalovirus immediate-early enhancer/chicken b-actin hybrid promoter [46]

Needle-type electrodes induced several smaller bone particles than plate-type electrodes in the targeted muscles. Needle-type electrodes require less electricity than plate-type electrodes (Figure 1C,D). The electric capacity of needle-type electrodes was represented in a two-dimensional area (Figure 1C), whereas that of plate-type electrodes could affect the three-dimensional area (Figure 1D). The electric field distribution might be an important factor in inducing uniformity over the plate compared with needle-type electrodes [47]. This may have contributed to the small particles of ectopic bone induced by needle-type electrodes in the target areas. However, some studies have reported that needle-type electrodes provide larger amounts of plasmid DNA in electroporation volumes than plate-type electrodes [48,49]. Therefore, *bmp-2* gene transfer to skeletal muscle with needle-type electrodes could be repeatedly performed. If small bone particles are separated, we can repeat gene transfer with needle-type electrodes to induce one-block bone formation after a clinical X-ray examination.

In future studies, we propose examining the time course of histological changes over an extended duration and measuring bone volume through three-dimensional and bone morphometric analyses. This will be carried out under various voltage conditions and with different needle electrode materials to assess biocompatibility.

5. Conclusions

bmp-2 gene transfer with a non-viral plasmid vector and needle-type electrodes could induce bone tissue formation in skeletal muscles with a 100% success rate. Thus, this approach could be reliable and safe for bone grafting with skeletal muscles.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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