The effect of electroacupuncture on osteotomy gap healing in a rat fibula model

Motohiro Inoue,1 Miwa Nakajima,1 Tatsuya Hojo,2 Megumi Itoi,3 Hiroshi Kitakoji1

ABSTRACT
Objective To examine the effect of electroacupuncture (EA) on osteotomy gap healing in a rat fibula model.
Methods A total of 40 12-week-old male Wistar rats underwent unilateral open osteotomy of the fibula to create a 2 mm gap. The rats were randomly assigned to an EA group (n=20) and a control group (n=20). In the EA group, a cathodal electrode was connected to an acupuncture needle percutaneously at the surgery site, while another acupuncture needle inserted 15 mm proximal to the surgery site was used as an anodal electrode. EA was performed 5 days a week for 6 weeks. The control group received no treatment. Some rats were killed at 3 days and 1 week after surgery and the cut end of the distal part of the fibula was surgically removed and histologically assessed. Haematoxylin and eosin (H&E) staining was used to measure total cell count and immunohistochemical staining to assess the increase in the bone morphogenetic protein 2 (BMP-2)-positive cells. The healing process was also assessed weekly after surgery via x-ray examinations.
Results At each time point, total cell count showed a marked increase in the EA group (p<0.05), while BMP-2 expression showed a tendency to increase in the EA group. Radiological examination showed a marked reduction in the distance between the cut ends of the fibula in the EA group.
Conclusions There was a marked increase in cell count and expression of growth factor in the EA group. These results indicate direct current EA could be useful for promoting bone healing.

INTRODUCTION
A large number of experiments on the promotion of fracture healing by electrical stimulation have been reported. Following this experimental technique, an invasive method of embedding electrodes has been used in clinical practice.1–3 Presently, when surgical treatment is performed, external fixators are frequently used as electrodes to provide electrical stimulation.4 5 However, the invasive nature of this procedure makes it inconvenient and risky, and it can be used for only a limited number of cases. In recent years, non-invasive methods that provide stimulation through the skin have been developed, such as low intensity pulsed ultrasound and pulsed electromagnetic fields, and their effectiveness has been reported.6 7 Nevertheless, while these methods eliminate the need for invasive procedures, applying stimulation to a local fracture site is difficult and the occurrence of ectopic ossification and attenuation of energy at the target site are matters of concern. In light of this situation, it is clear that establishing a method of bone healing promotion in which stimulation can be applied less invasively to a limited location would enable wider application of electrical stimulation.

In a previous study, we examined the effect of intermittent direct current electrical stimulation in a rat tibial osteotomy model and verified that the stimulation promoted bone healing.8 Using acupuncture needles as electrodes has the advantages of eliminating the need for an invasive procedure and enabling selective stimulation of deeper regions, making simple, quick and frequent applications possible. The aim of this study was to clarify the effect of electroacupuncture (EA) stimulation on osteotomy gap (bone defect) healing in a rat fibula osteotomy model in which there was no contact between the cut ends of the fibula, a generally unfavourable condition for fracture healing.
METHODS

Animals
A total of 40 Wistar rats (12-week-old males weighing 250–300 g) were used as experimental animals. Under anaesthesia with pentobarbital (50 mg/kg) administered intraperitoneally, the fibula on the right side was exposed and an osteotomy was used to make a 2 mm osteotomy gap in the shaft of the fibula 20 mm above the lateral malleolus. After making the osteotomy gap, the skin was sutured with 3-0 nylon without fixing the bone to create a fibula osteotomy model. The rats were randomly assigned to two groups. Antibiotics (enrofloxacin; Bayer Medical, Osaka, Japan) were given for 3 days after surgery, and the surgical wound was cleaned with povidone-iodine solution every other day for 3 weeks after surgery. Except during examinations, the rats were allowed to eat, drink and move freely inside an optimally conditioned cage. This study was conducted with the approval of the Experimental Animal Ethics Committee of Meiji University of Integrative Medicine (authorisation no. 23-4).

Experimental groups
EA group (n=20): starting the day after creating the model, intermittent direct EA was performed 5 days/week for 6 weeks using an acupuncture needle inserted at the cut end of the distal part of the fibula as a cathode and another at a site 15 mm proximal to the cathode as an anode. Both needles (30 mm in length, 0.24 mm in diameter, stainless steel; Seirin, Shizuoka, Japan) were inserted percutaneously with the tip of the cathode touching the periosteum and the anode inserted into the anterior tibial muscle layer. The EA method was the same as that applied in a previous study with a rat tibial osteotomy model. Using an electric stimulator (SEN-3301; Nihon Kohden, Tokyo, Japan) and isolator (SS-104J; Nihon Kohden, Tokyo, Japan), 5 ms square pulses of 50 Hz were delivered at 20 μA for 20 min.8 EA was performed with the rats lightly anaesthetised with pentobarbital (50 mg/kg, 0.1 ml) administered intraperitoneally and with all four limbs restrained.

For the control group (n=20), after surgery, the rats were anaesthetised and restrained for the same periods of time as rats in the EA group.

Assessment
Radiographic evaluation (n=10 in each group) was performed. From the day of creating the model, the rats were anaesthetised with pentobarbital (50 mg/kg) administered intraperitoneally and radiographs were taken weekly for 6 weeks. Lateral images were evaluated and the width of the gap between the proximal and distal fibular stumps was measured with digital callipers (CD-20PSX, Mitutoyo, Kawasaki, Japan).

Histological evaluation (n=5 in each group on each date of evaluation) was also performed 3 days and 1 week after surgery; sections of fibula were removed from the rats and fixed in buffered formalin solution for 7 days followed by decalcifying with EDTA and paraffin embedding using the standard method. Then, 4 μm sagittal sections were made, stained with haematoxylin and eosin (H&E) and immunohistochemical stain, examined with a light microscope (Nikon E600: Nikon Corporation, Tokyo, Japan) and photographed with a digital camera for microscopy (Nikon Dxm1200: Nikon Corporation). The images were then stored on a PC. Quantitative evaluation of H&E-stained samples was performed using image analysis software (Image J V1.42: National Institutes of Health, Bethesda, Maryland, USA),9 and the total number of cells was counted using H&E staining included inflammatory cells, mesenchymal cells, fibroblasts and osteoblasts, within a set region of interest (0.43×0.34 mm² corner of the cut end of bone).

After deparaffinisation, tissue sections were stained using the standard method (H&E staining).

Immunohistochemical staining
Sections were immersed in 0.01 M citrate buffer solution (pH 6.0) and antigen activation was performed in an autoclave at 121°C for 15 min. Sections were then treated in 0.3% H₂O₂ solution for 5 min to block endogenous peroxidase activity, and then washed in 0.05 M phosphate buffered saline (PBS; pH 7.6). Following treatment at room temperature with a protein blocking agent for 5 min, anti-human bone morphogenetic protein-2 (BMP-2) mouse monoclonal antibody (#ab6285: Abcam, Tokyo, Japan) at a dilution of 1:1000 was used as a primary antibody, and samples were left at 4°C to react overnight. After washing with PBS, anti-mouse horseradish peroxidase (HRP)-conjugated Envision antibodies were used as secondary antibodies (#K4001: DAKO Cytomation, Glostrup, Denmark), and left to react for 30 min. The colour was developed using 3,3'-diaminobenzidine-4HCl and nuclear staining was performed with Mayer’s haematoxylin. Assessment of the expression in the BMP-2-positive cells were completed by a coauthor who was unaware of group allocation.

Statistical analysis
All values presented are means±SD. The t test was used to compare the total cell counts of each group measured at each evaluation time point using H&E stains. Changes in the bone gap width in the two groups over the study period were analysed using repeated measures analysis of variance. The between-group comparisons on gap width at each evaluation time point were tested using the Student t test and Bonferroni’s adjustment. Statistical analyses were performed using Statview V4.5 (SAS Institute, Tokyo, Japan) and data were considered significant when the p value was less than 0.05.
RESULTS

Radiographic evaluation

The change over time in the bone defect (gap width) in each group is shown in Table 1. Typical soft x-ray findings are shown in figure 1. Interaction was observed between the group over time (p < 0.0001). The gap grew larger over time in the control group and shorter in the EA group. In addition, at each weekly evaluation of gap width with soft x-rays taken from weeks 1 to 6, there was a significant difference between the two groups from 2 weeks onward (2 weeks: p < 0.001, 3–6 weeks: p < 0.0001) (figure 2). At the final evaluation 6 weeks after surgery, no bone healing was observed in any of the control group rats, whereas it was observed in 3 of 10 limbs in the EA group.

Histological evaluation

The results of quantitative analysis of total cells counts using H&E stain are shown in Table 2 and figure 3. At 3 days and 1 week after surgery, a significant difference was observed between the two groups, with an increased number of cells in the EA group (p < 0.05). Figure 4 shows typical H&E stained tissue images. At 3 days and 1 week after surgery, compared with the control group, in the EA group there were a large number of cells including inflammatory cells, mesenchymal cells, fibroblasts and osteoblasts near the bone stump.

Figure 5 shows typical images of tissue stained with BMP-2 immunohistochemical stain. In both groups, compared with after 1 week, at day 3 strong cell staining of what are thought to be bone cells and osteoblasts can be seen, and at both evaluation days, antigen–antibody expression also tended to be stronger in the EA group.

DISCUSSION

In a previous study using a rat tibia fracture model, we reported that EA could possibly promote bone healing.8 In the study, we also found that simple insertion of the acupuncture needle without electrical stimulation had no effect on the bone healing process. We therefore considered it is unnecessary to insert needles in the control rats. Using acupuncture needles as electrodes eliminates the invasive procedures required to embed/remove electrodes and allows flexibility in selecting a stimulation site. It also enables localised electrical stimulation of deeper regions when the promotion of bone healing is required. With these and other advantages, EA could be anticipated to be a simple useful method of treatment to promote bone healing. This study was conducted to obtain more data on the effect of EA stimulation on bone healing.

Table 1 Change over time of bone defect (gap width)

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone defect created (mm)</th>
<th>1 week (mm)</th>
<th>2 weeks (mm)</th>
<th>3 weeks (mm)</th>
<th>4 weeks (mm)</th>
<th>5 weeks (mm)</th>
<th>6 weeks (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA group</td>
<td>2.0±0.0</td>
<td>1.94±0.19</td>
<td>1.19±0.50</td>
<td>0.76±0.54</td>
<td>0.57±0.56</td>
<td>0.51±0.55</td>
<td>0.48±0.54</td>
</tr>
<tr>
<td>Control group</td>
<td>2.0±0.0</td>
<td>1.97±0.11</td>
<td>1.97±0.28</td>
<td>2.07±0.34</td>
<td>2.20±0.43</td>
<td>2.28±0.62</td>
<td>2.34±0.70</td>
</tr>
</tbody>
</table>

A significant interaction (time×group) with repeated measures analysis of variance was found between the two groups (p < 0.0001). Data are expressed as mean±SD.

Figure 1 Typical soft x-ray images at each evaluation date.
In addition, with the aim of using EA for a wider range of bone healing applications (e.g., as a method of bone lengthening), we conducted histological and radiological examinations to determine the effect of EA in a rat fibula model in which there was no contact between the cut ends of the bone at the osteotomy site.

In terms of change over time in the width of the gap, soft x-ray images showed that the gap became larger in the control group with no bone healing observed in any of the rats, while it became smaller in all cases in the EA group and bone adhesion was observed in 3 of 10 limbs. These results clarify that direct current EA with the cathode at the site of bone defect promoted bone formation. Histological evaluation with H&E staining also showed that at 3 days and 1 week after surgery, there was a significant difference between the two groups, with an increase in the number of cells observed at the cut end of the distal part of the fibula where the cathode was located. In this study, as well as osteoblasts the cells counted using H&E staining included inflammatory cells, mesenchymal cells, fibroblasts and osteoblasts, which all showed a relative increase in the EA group. Immunohistochemical staining also showed a difference in BMP-2 expression between the two groups at

**Table 2** Difference between the two groups in the number cells counted using haematoxylin and eosin (H&E) stain at each evaluation date

<table>
<thead>
<tr>
<th>Group</th>
<th>3 days</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA group (no.)</td>
<td>42.1±23.7</td>
<td>63.6±22.8</td>
</tr>
<tr>
<td>Control group (no.)</td>
<td>27.5±17.5</td>
<td>31.8±2.7</td>
</tr>
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</table>

The difference between the two groups in the number cells counted using H&E staining included inflammatory cells, mesenchymal cells, fibroblasts and osteoblasts at each evaluation date is shown. Data are expressed as mean±SD.

**Figure 2** Comparison of gap width in the two groups at each evaluation date. *p<0.001, **p<0.0001 versus control.

**Figure 3** Quantitative evaluation of total cell counts using haematoxylin and eosin (H&E) staining included inflammatory cells, mesenchymal cells, fibroblasts and osteoblasts at 3 days (left) and 1 week (right) after surgery. *p<0.05 versus control.
3 days and 1 week, and stronger staining of BMP-2 was seen in the EA group. From this, it is thought that the mechanism of action for the promotion of bone formation and adhesion when using the fracture or defect site as the cathode during direct current EA stimulation involves reduced oxygen partial pressure.
and increased hydrogen ion concentration at the cathode site,\textsuperscript{10} which creates ideal conditions for bone adhesion. In addition, associated cell differentiation, proliferation and migration due to the increased expression of growth factors that are required for bone formation, such as BMPs, may also be involved. It has been reported that one of the growth factors, the BMP that was used as a marker in this study, is strongly expressed during the early stage of fracture healing and could be involved in cell differentiation.\textsuperscript{11} It is known that during the early stage of healing, BMP-2 in particular has an inductive action on bone and cartilage.\textsuperscript{12} Further examination of other growth factors is necessary, but the tendency towards increased expression of BMP-2 in the EA group suggests that the differentiation of undifferentiated mesenchymal cells may be involved in the promotion of bone formation by EA. Furthermore, since strong expression of BMP-2 was observed during the early stage of bone healing, which might reflect the effect of EA, when using EA as a method to promote bone healing starting treatment at an early stage of the healing process could be an important factor in achieving better results.

The results of this study using a bone defect model and a previous study using a rat tibia fracture model\textsuperscript{1} clearly show that, in many cases, EA stimulation is an effective method for promoting bone healing. However, before its clinical application, the extent of the electrical effects of EA, as well as the optimal conditions to prevent adverse events, should be examined. Once these have been clarified, EA could be used in the clinical setting.

CONCLUSIONS

Using a rat fibula model, we conducted radiological and histological studies of the effect of intermittent direct current EA stimulation on bone healing in cases where the bone ends at the fracture site do not touch. During the initial stage after surgery, an increase in the number of cells and strong expression of growth factors were observed on the cathode side in the EA group. As a result, complete bone healing was obtained in several cases. We propose that EA is a useful method for promoting bone formation and adhesion at bone fracture and defect sites.

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Contributors MI: designed the study, conducted research, performed data analysis and wrote the manuscript. MN: conducted research and was involved with acquisition of data. TH: was involved with analysis and interpretation of data. MI: was involved with data analysis and supervision of the study. HK: revised the article critically for important intellectual content and had overall control of the study.

Competing interests None.

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