Effect of acupuncture therapy on fracture healing in rats with femur fractures

Seval Yilmaz, Emre Kaya, Erhan Yilmaz, Ahmet Kavakli, Suleyman Gurbuz, Mustafa Ozkaraca

OBJECTIVE: To investigate the effect of acupuncture therapy on fracture healing in rats with femur fractures.

METHODS: A total of 10 groups were formed; control group, groups sacrificed on 7th, 14th, and 21st days of fracture formation, groups to which acupuncture was applied for 7, 14, and 21 d, groups to which fracture and acupuncture were applied for 7, 14, and 21 d. A transverse fracture line was formed in femurs of rats by using a Gigli saw. The Kirschner wire was driven retrograde down from the fracture line to proximal part of the bone and then, the fracture was fixed towards distal part. Acupuncture was applied to the rats for 7, 14, and 21 d as 4 sessions per week after formation of the fracture.

RESULTS: Malondialdehyde (MDA), reduced glutathione (GSH) levels, catalase (CAT), glutathione-S-transferase (GST), superoxide dismutase (SOD), and glucose-6-phosphate dehydrogenase (G6PD) activities were measured. Despite the increased MDA levels, G6PD and SOD activities reduced during the fracture healing. There was a statistically significant increase in MDA, GSH levels, and G6PD activity in fracture groups compared to control group, but CAT, GST, and SOD activities decreased. The use of acupuncture enhanced callus development and bone mineralization during bone healing.

CONCLUSION: The acupuncture therapy can affect suppression of the effects of free oxygen radicals and regulation of the antioxidant enzyme activity in fracture healing. Thus, it is suggested that acupuncture treatment would be beneficial for fracture healing in order to eliminate the negative effects induced by oxygen free radicals.

INTRODUCTION

Bone fracture healing is a complex process which results in full recovery of function and structure of injured bone tissue, but all the mechanisms get involved in this process and their mutual interaction is not exactly understood. The fracture site is structurally unstable and hypoxic due to severe bone loss and ischemia. The fracture healing process can be divided into three phases: early in-
Inflammation at the acupuncture site, decreased inflammation, release of endorphin, recovery of muscle stiffness, increased microcirculation of brain, decrease of high blood pressure and lipid concentration, reduced hypersensitivity of skin and mucous membranes to various factors, analgesia and hypoalgesia, stimulation of anterior or pituitary hormone release, increased immune response and resistance to bacterial infections, stimulation of lipolysis via a secondary mechanism, and hypnotic effects are observed.

There are many studies reporting that acupuncture accelerates the process of fracture healing. Su et al.\(^6\) have reported that acupuncture can accelerate the healing of osteoporotic fracture and is clinically better when combined with acupuncture medications to relieve fracture symptoms compared to medication. Barker et al.\(^1\) found that auricular acupuncture applied at three specific points was effective in reducing pain and anxiety in elderly patients who had a hip fracture. Yang\(^2\) reported that acupuncture had a certain effect on stimulating the callus formation in early and middle phases but no effect in increasing of the bone volume in rabbits.

In the present study, it was hypothesized that the acupuncture therapy in early period following formation of fracture could influence fracture union positively as well as its effect on free oxygen radicals.

**MATERIALS AND METHODS**

Seventy Wistar-Albino female rats were divided as control and experimental groups: control group (group \(\text{I}\)), group sacrificed on 7th day of fracture formation (group \(\text{II}\)), group sacrificed on 14th day of fracture formation (group \(\text{III}\)), group sacrificed on 21st day of fracture formation (group \(\text{IV}\)), group to which acupuncture was applied for 7 d (group \(\text{V}\)), group to which acupuncture was applied for 14 d (group \(\text{VI}\)), group to which acupuncture was applied for 21 d (group \(\text{VII}\)), group to which fracture and acupuncture were applied for 7 d (group \(\text{VIII}\)), group to which fracture and acupuncture were applied for 14 d (group \(\text{IX}\)), and group to which fracture and acupuncture were applied for 21 d (group \(\text{X}\)). The protocol for the use of animals was approved by the Firat University Animal Experiments Local Ethics Committee (Protocol No. 2011/07-92).

General anesthesia was applied to the rats. In the operation, lateral femoral incision (at 2 cm long) was made and the femur was separated through the thigh anterolateral intermuscular septum. A mid-shaft osteotomy was performed using a Gigli saw and fixed with a 1.2-mm diameter stainless steel Kirchner wire passing retrograde into the proximal fragment and then into the medullary canal of the distal fragment. The incisions were sutured. Weight bearing was not restricted or the use of the operated limb was not applied. The fracture formation was confirmed radiographically based on direct radiographs. Radiographs were ob-

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\(^1\) Barker, et al. 2011.  
\(^2\) Yang, et al. 2011.  
\(^7\) Yang, et al. 2011.  

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Oxidative stress was measured as thiobarbituric acid reactive substance (TBARS) level, which was measured according to the method of Ohkawa et al.9 The TBARS level was expressed as nmol/g tissue.

Biochemical analysis

The rats were treated postoperatively without any complication and did not show any sign of discomfort. Table 2 shows bone MDA, GSH levels, CAT, GST, SOD, and G6PD activities. There was no statistically significant difference between acupuncture groups in terms of bone MDA and GSH levels, CAT, GST, SOD and G6PD activities compared to the control group. A statistically significant increase was determined between bone MDA and GSH levels of all fracture groups compared to the control group (P < 0.05). Increased MDA level was more distinct on 7 and 14th days of fracture in comparison with the day 21. A significant decrease was observed in fracture + acupuncture groups compared to fracture groups (P < 0.05). Bone GSH levels were higher on days 14 and 21 of the fracture than the day 7. There was a significant decrease in fracture + acupuncture groups compared to fracture groups (P < 0.05). Bone CAT, GST and SOD activities showed a statistically significant decrease in fracture groups compared to the control group (P < 0.05). A significant increase was observed in fracture + acupuncture groups compared to the fracture group. GSH-Px activity was at immeasurable level in bone tissue.

RESULTS

Biochemical analysis

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Radiological analysis
In general fracture healing, identifying the formation of bony callus is the radiographic sign used to evaluate the stage and rate of healing (Figure 1). Therefore, the callus tissue area was calculated. It was not assessed because there was no difference between the results of the day 7. It was found that while total callus area on day 14 of the fracture was (23.4 ± 2.6) mm², total callus area of the fracture + acupuncture administration was (31.4 ± 3.2) mm². While total callus area on day 21 of the fracture was (24.9 ± 2.9) mm², total callus area on day 21 of fracture + acupuncture administration was (73.2 ± 4.0) mm² (Figures 2, 3).

On day 7, a callus with a massive fibrous tissue containing a small amount of immature chondrocytes was observed in the fracture group and fracture + acupuncture group. There were new vein occurrences. There was no statistically significant difference between the groups. On day 14, a total of 7 rats in the fracture group had an equal callus with fibrous and cartilaginous tissue; whereas, in fracture + acupuncture group, 7 rats showed fibrous and cartilaginous tissue in equal amounts and 4 rats had cartilage containing heavy cartilage and a slight fibrous tissue. A statistically significant difference was found between the groups. On day 21, 7 rats in the fracture group showed cartilage and immature bone tissue at equal rate, while the fracture + acupuncture group showed a callus containing 4 masses of immature bone and a small amount of cartilaginous tissue. A statistically significant difference was

Table 1 The scoring system used to assess histological healing of fractures

<table>
<thead>
<tr>
<th>Score</th>
<th>Tissue formation</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Fibrous tissue</td>
</tr>
<tr>
<td>2</td>
<td>Weighted fibrous tissue</td>
</tr>
<tr>
<td>3</td>
<td>Fibrous and cartilaginous tissue in equal amounts</td>
</tr>
<tr>
<td>4</td>
<td>Weighted cartilage and minimal amount of fibrous tissue</td>
</tr>
<tr>
<td>5</td>
<td>Cartilage</td>
</tr>
<tr>
<td>6</td>
<td>Weighted cartilage and small amount of immature bone</td>
</tr>
<tr>
<td>7</td>
<td>Cartilage and immature bone tissue equal amounts</td>
</tr>
<tr>
<td>8</td>
<td>Weighted immature bone and small amount of cartilage tissue</td>
</tr>
<tr>
<td>9</td>
<td>Fracture healing with immature bone</td>
</tr>
<tr>
<td>10</td>
<td>Fracture healing with mature bone</td>
</tr>
</tbody>
</table>

Table 2 MDA, GSH levels, CAT, GST, SOD and G6PD activities in bone tissue of fractured rats

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/g protein)</th>
<th>GSH (µmol/mL)</th>
<th>CAT (µmol/g protein)</th>
<th>GST (U/g protein)</th>
<th>SOD (U/g protein)</th>
<th>G6PD (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67.15±1.412a</td>
<td>0.23±0.011a</td>
<td>18.31±3.323b</td>
<td>105.36±2.200b</td>
<td>53.13±1.603a</td>
<td>1.19±0.041b</td>
</tr>
<tr>
<td>Fracture (7d)</td>
<td>96.02±2.581a</td>
<td>0.79±0.055a</td>
<td>10.22±3.312b</td>
<td>81.56±4.672ab</td>
<td>41.15±1.582b</td>
<td>2.77±0.146b</td>
</tr>
<tr>
<td>Fracture (14d)</td>
<td>103.00±2.763a</td>
<td>1.08±0.075a</td>
<td>11.70±0.082b</td>
<td>64.67±2.795a</td>
<td>41.10±1.731a</td>
<td>2.06±0.217a</td>
</tr>
<tr>
<td>Fracture (21d)</td>
<td>86.83±3.594a</td>
<td>0.88±0.093a</td>
<td>10.61±3.535a</td>
<td>71.31±3.493ab</td>
<td>40.90±1.386a</td>
<td>2.79±0.642a</td>
</tr>
<tr>
<td>Acupuncture (7d)</td>
<td>74.15±2.444a</td>
<td>0.40±0.044a</td>
<td>18.16±1.011a</td>
<td>97.10±1.142a</td>
<td>50.25±5.681a</td>
<td>0.66±0.023a</td>
</tr>
<tr>
<td>Acupuncture (14d)</td>
<td>66.67±3.592a</td>
<td>0.27±0.012a</td>
<td>17.06±1.503a</td>
<td>84.45±14.401b</td>
<td>50.10±6.630a</td>
<td>1.43±0.115a</td>
</tr>
<tr>
<td>Acupuncture (21d)</td>
<td>72.25±5.664a</td>
<td>0.25±0.013a</td>
<td>17.86±0.532a</td>
<td>91.14±2.473a</td>
<td>48.40±2.073b</td>
<td>0.59±0.017a</td>
</tr>
<tr>
<td>Fracture + Acupuncture (7d)</td>
<td>73.57±3.801a</td>
<td>0.41±0.024a</td>
<td>16.32±0.713a</td>
<td>103.81±3.016a</td>
<td>53.72±3.632a</td>
<td>1.86±0.091a</td>
</tr>
<tr>
<td>Fracture + Acupuncture (14d)</td>
<td>74.42±1.341a</td>
<td>0.46±0.071a</td>
<td>17.00±0.461a</td>
<td>105.29±1.245a</td>
<td>57.30±3.283a</td>
<td>1.71±0.063a</td>
</tr>
<tr>
<td>Fracture + Acupuncture (21d)</td>
<td>69.43±1.162a</td>
<td>0.44±0.015a</td>
<td>16.56±0.846a</td>
<td>104.37±4.066b</td>
<td>51.56±4.574a</td>
<td>1.42±0.333b</td>
</tr>
</tbody>
</table>

Notes: control: group without any application. Fracture (7d): group sacrificed on 7th day of fracture formation. Fracture (14d): group sacrificed on 14th day of fracture formation. Fracture (21d): group sacrificed on 21st day of fracture formation. Acupuncture (7d): group to which acupuncture was applied for 7. Acupuncture (14d): group to which acupuncture was applied for 14 d. Acupuncture (21d): group to which acupuncture was applied for 21 d. Fracture + acupuncture (7d): group to which fracture and acupuncture were applied for 7 d. Fracture + acupuncture (14d): group to which fracture and acupuncture were applied for 14 d. Fracture + acupuncture (21d): group to which fracture and acupuncture were applied for 21 d. MDA: Malondialdehyde; GSH: glutathione; CAT: catalase; GST: glutathione-S-transferase; SOD: superoxide dismutase; G6PD: glucose-6-phosphate dehydrogenase. The data are expressed in mean ± standard error of mean for seven animals per group. Within rows, means with different letters (a, b, c and d) are significantly different (P < 0.05).
found between the groups (Table 3, Figure 4). As a result, fracture + acupuncture group was more advanced than fracture group on the 14th and 21st days (P < 0.05).

DISCUSSION

The factors affecting fracture healing and acceleration of healing are among popular topics researchers have investigated. When early periods of fracture healing in human have been viewed, the rate of repair seen in diaphyseal fractures is similar to the repair rate of experimental fracture. Therefore, the results obtained from experimental fracture models are thought to be adapted to human beings. Based on the findings of recent studies, free oxygen radicals are also among the factors affecting fracture healing. Reduced blood flow to a fracture results in regional ischemic injury, and in the peripheral regions of the "ischemic zone", there are viable cells that can be salvaged if managed adequately. Otherwise, these cells undergo irreversible injury. This phenomenon is referred as reperfusion injury and may reduce bone healing. Similarly, the first 3 d of fracture healing may be compared with the ischemic period. Thus, during this period, no oxidative stress injury occurs. Then, in the stage of callus formation, fibroblast and collagen cells as well as new capillary vessels with other inflammatory cells increase the production of oxygen free radicals, which may cause oxidative injury in fractured bones, as seen in the other tissues with reperfusion injury. Enhanced osteoclastic activity observed in bone disorders may have been responsible for the increased production of ROS in the form of O$_2^.-$. One of the most damaging ef-
Effects of ROS is lipid peroxidation, the end product of which is MDA which also serves as a measure of osteoclastic activity. Enhanced osteoclastic activity observed in bone disorders may have been responsible for the increased production of ROS in the form of O$_2^•$ which becomes evident by increased levels of MDA levels. One of the most detrimental effects of ROS is lipid peroxidation, the end product of which is MDA which also serves as a measure of osteoclastic activity.

ROS concentration occurring in tibial fractures concomitant with temporary ischemia and reperfusion was determined to be considerably high in inflammation stage of fracture healing. Gokturk indicated that ROS produced by leucocytes in the body negatively influenced structural or functional integrity of cartilage and peripheral joint structures as well as fracture healing. As a result of their examinations on culture bones, Garett et al. showed that ROS and particularly superoxide played a mediator role in osteoclast formation and activation and thus in bone resorption. Norazlina et al. reported that free radicals had a cytotoxic impact on osteoblasts.

The increased levels of MDA, peaking by the 14th day, showed a significant level of oxidative stress during the 2nd week after the fracture. These data suggested that the production of oxygen free radicals during fracture healing was the greatest in the inflammation period. The fact that MDA values in the experimental group were still significantly different from the control group on the following days indicated that the production of free radicals also continued during the repair period although it was low compared to the inflammation period. Excessive and prolonged ROS activity causes tissue damage which impedes osteoblast differentiation and instigates long healing times. Results of the present study were compatible with study by Gokturk, which evaluated oxidant status during bone healing in rats by using MDA levels in bone specimens as an indicator of oxidative stress. Furthermore, Prasad et al. pointed out that values of MDA did not almost change in first 7 d after femur fracture and then increased in 2nd and 3rd weeks after the fracture. They also showed that oxidative stress was proportional to number of fractured bones, so it was much higher in multiple fractures. One of the proposed mechanisms of unchanged values of MDA and thus oxidative stress are ischemic in region of fracture. Furthermore, during the healing process, callus begins to form and produce new cells, which include new capillary vessels. The increase in vascularization in this region and the increase in inflammatory cells lead to the increased production of oxygen free radicals. In the study conducted by Durak et al. on femoral fractures of rabbits, they determined that

<p>| Table 3 Histopathological evaluation in bone tissue of fractured rats |
|------------------------|------------------------|------------------------|</p>
<table>
<thead>
<tr>
<th>Item</th>
<th>Fracture group</th>
<th>Fracture+acupuncture group</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 d</td>
<td>2.1±0.4 $^a$</td>
<td>2.3±0.5 $^a$</td>
</tr>
<tr>
<td>14 d</td>
<td>3.0±0.0 $^a$</td>
<td>3.6±0.5 $^a$</td>
</tr>
<tr>
<td>21 d</td>
<td>7.0±0.0 $^a$</td>
<td>7.6±0.5 $^a$</td>
</tr>
</tbody>
</table>

Notes: fracture (7 d): group sacrificed on 7th day of fracture formation. Fracture (14 d): group sacrificed on 14th day of fracture formation. Fracture + acupuncture (7 d): group to which fracture and acupuncture were applied for 7 d. Fracture + acupuncture (14 d): group to which fracture and acupuncture were applied for 14 d. Fracture + acupuncture (21 d): group to which fracture and acupuncture were applied for 21 d. The data are expressed in mean ± standard error of mean for seven animals per group. Within rows, means with different letters (a, b, c, d and e) are significantly different (P < 0.05).

Figure 4 View of bone tissue in rats by light microscope
A: fracture group on 7th day: weighted fibrous tissue and new vessel formation in the immune chondrocytes in the fracture group on 7th day. A [(hematoxylin-eosin (HE)) and A1 [(masson trichrome (MT)] (magnification ×20); B: fracture + acupuncture group on 7th day: weighted fibrous tissue and new vessel formation (arrow) containing mature chondrocytes in the fracture + acupuncture group on 7th day. B (HE staining) and B1 (MT staining, ×20); C: fracture group on 14th day: equal amounts of fibrous and cartilaginous tissue formation in the fracture group on 14th day. C (HE staining) and C1 (MT staining) (magnification ×20); D: fracture + acupuncture group on 14th day: aggregate cartilage tissue formation in the fracture + acupuncture group on 14th day. D (HE staining) and D1 (MT staining, ×20); E: fracture group on 21st day: cartilage and immature bone formation in the fracture group on 21st day (HE staining) (magnification ×20); F: fracture + acupuncture group on 21st day: immature bone formation in the fracture + acupuncture group on 21st day (HE staining) (magnification ×20).
the effects of free oxygen radicals in control group were stronger in hematoma fluid of fracture than plasma. This was associated with ischemia and reperfusion by resulting in increased lipid peroxidation developing at fracture site. Antioxidant enzymes (GSH, SOD, CAT and GST) provide the internal defense against free radicals and may neutralize them before they are able to attack membrane and cause damage. It is important to examine their values during the healing and thus their role in fracture. Values of all examined parameters of anti-oxidative defense system showed a statistically significant change in measurement points. One mechanism for removal of $\cdot O_2$ is through the protective $O_2^-$ scavenging enzyme SOD. It was found that SOD activity decreased in the fractured femur of rats. SOD was likely used to overcome the free radicals released during the early phase of fracture healing. The main free radicals released during the early phase of fracture healing were $O_2^-$ radicals. These free radicals were also generated by osteoclasts, which are the cells responsible for digesting the bone debris during the early phase of fracture healing. The $H_2O_2$ was in turn degraded by GSH-Px and CAT. The GSH-Px activity could not be measured but there were two antioxidant enzymes working together to degrade $H_2O_2$. CAT activities significantly decreasing in the fracture of experimental rats observed at phase of healing suggested that $H_2O_2$ accumulation might not be properly neutralized by CAT. This decrease in the antioxidant enzymes might indicate the enzyme adaptive against higher oxidative stress in the fractured rats. In other words, these results may indicate the role of decreased anti-oxidative defense system in pathogenesis of femoral fractures. Inhibition of the antioxidant enzyme activities such as SOD and CAT was found to increase $O_2^-$ production by the osteoclasts represented by increased levels of MDA. Therefore, oxidative stress caused by decreased anti-oxidative defense could be an important contributor for morphological and functional changes in bone. Oxidative stress is an important mediator of bone loss since the antioxidant deficiency has been found to be more common in the bone fracture. It has been known for a certain period of time that tissue damage can induce a high yield of ROS that can lead to an imbalance between an excessive generation of oxidant compounds and insufficient antioxidant defense mechanisms. In this study, fracture force of fracture group was found to be significantly lower than the control group and this was associated with high levels of ROS effect just as stated by Cetinbas et al. X-ray findings showed significantly better fracture healing with acupuncture treatment compared to the control group. The bone mineral density of the callus tissue that is developing within the fracture line can be measured non-invasively and reflects the quality of new callus tissue. In the study by Balogh et al. the fibula of adult rats were cut at the mid-diaphysis with scissors and examined at various stages of repair. Seven days after the fracture, a bulky cartilaginous callus developed. As the fibroblast-like osteoprogenitor cells of the callus developed into chondrocytes, G6PD enzyme activity increased. The enzyme pattern of the callus was essentially the same on days 14 and 7. Avitabile et al., Basu et al., Cohen et al., and Leveille et al. reported that antioxidant system reduced and there was a correlation between increase of oxidative stress and decrease of bone density. Lipid peroxidation was reported to increase bone resorption by directly activating osteoclasts. The most important matter in fracture healing is the duration of union. Drugs to accelerate the union may have adverse effects. Steroids, which are frequently used for immune system disorders today, likely have negative effects on fracture healing in long-term use. One of the most crucial problems of trauma surgery is infections. Infection particularly occurring after open fractures both inhibits fracture healing and may lead to systemic issues. The use of antibiotics is necessary for such kind of injuries. Here, antibiotics also have negative effects on fracture healing. Huddlestone et al. identified that the rate of resorption in rats receiving Cefazolin after femoral fracture was similar to control group. The latest resorption was determined in ciprofloxacin group. Gentamicin, one of the most frequently used antibiotics, was also observed to inhibit fracture healing. Mammi et al. showed that electromagnetic stimulation had positive effects on recovery of femoral and tibial osteotomies. Another study investigated the effects of acupuncture on healing process of tibial fracture in rats. Unilateral open osteotomy was performed on tibias of 12 week-old rats. While a 15-mm acupuncture needle was pricked to proximal fragment of surgical site as anodal electrode in acupuncture group, an acupuncture needle penetrating directly into percutaneous surgical site was inserted as cathodal electrode and acupuncture (50 Hz, 20 uA, 20 min) was applied daily for 3 weeks. Accelerated bone healing and callus were observed in acupuncture group. The 6th week displayed an excellent result for acupuncture group also in mechanical test and no difference was found between sham and control groups. The use of acupuncture increased development of callus and bone mineralization during bone recovery period. It was reported that duration of treatment was shorter and mobility functions were much better owing to acupuncture application. In a study, ovariotomy was performed to induce osteoporosis in rats and left femurs of rats were broken openly 3 months after ovariotomy. Left hind legs of the rats in the experimental group were treated daily with "Huantiao" (GB 30), "Zusanli" (ST 36), "Yanlingguan" GB 34), and "Weizhong" (BL 40) acupuncture points. Recovery process of fracture in acupuncture groups was faster. Neurotrophic factor of brain origin and tyrosine kinase B was lesser in callus of osteoporotic fracture than normal fracture and acupuncture was
suggested to accelerate healing process of fracture.31 In another study; subcutaneous implantation of golden wire into acupuncture points was showed to have long-term effects on bone regeneration in ulna bone defects in rats.42 Therapeutic effects of acupuncture with indurate hook on nonunion were investigated and acupuncture along with indurate hook was suggested to be a good method for inducing fracture healing. Acupuncture was used for 30-40 d in radius and ulna fractures in people with algodystrophy, treatment duration was much shorter, and mobility functions were much better. 

Acupuncture supplementation significantly decreased the MDA level during fracture healing compared to the control group. It was concluded that acupuncture applied immediately after formation of fracture would have positive effects to improve fracture healing. The bone GSH levels decreased in association with acupuncture administration compared to the control group. This may be explained by the reduced need for endogenous antioxidants due to the effect of acupuncture. Acupuncture begins to show its beneficial effects in the first week of treatment. Since the level of oxidative stress is mostly prominent during the early phases of fracture healing, acupuncture administration seems to be the most beneficial during this period after the fracture. Another important result of this study is the effects of acupuncture on both fracture healing and the oxidant-antioxidant system.

In conclusion, this study consistently showed beneficial effects of acupuncture on fracture healing as assessed by several tools including bone mineral density measurements and radiographic evaluations. These results were supported by the changes in the level of endogenous molecules in anti-oxidative process compared to the control group. This indicated a potential role for acupuncture in the treatment of fractures through the facilitation of healing and union. It is thought that acupuncture has the potential to be used systemically or locally under many conditions in the future. Thus, it is required to conduct further studies examining the effect of this substance in clinical settings.

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