

# Can local administration of humic acid shorten recovery time of mandibular fractures? Experimental study

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## Abstract

**Objective:** The aim of the present pilot study was to evaluate the effects of a single local dose administration of humic acid on healing of subcondylar mandibular fractures in rats.

**Methods:** In this study, a randomized experimental protocol was used. The study was conducted with 16 male Wistar-albino rats that were 16–18 weeks old. The rats (n=16) were randomly divided into two groups: Group HA received humic acid (0.3 cc/site, n=8) and Group C received no additional medical administration (control group, n=8). A full-thickness surgical osteotomy was performed in the subcondylar area. A single dose of humic acid (0.3 cc/site) was administered locally by spraying on the bone surfaces of the fracture line. Mandible was dissected on post-operative day 21. Then, fractured hemimandibles were obtained for histopathological examination.

**Results:** The median score of bone fracture healing was 7.16 (range: 7 to 8) in the Group HA and 7.50 (range: 7 to 8) in the Group C. When the groups were compared in terms of bone healing scores, there was no statistical difference between the Group HA and the Group C (p>0.05).

**Conclusion:** Results of this study showed that local administration of humic acid was not efficient for healing of bone fractures. However, we are of the opinion that it is required to conduct more comprehensive studies, including humic acid's different concentrations and administration manners, evaluating the effects of humic acid on tissue both histopathologically and in terms of inflammatory and proinflammatory cytokine levels.

**Keywords:** Humic acid, subcondylar mandibular fractures, fracture healing.

## Özet: Lokal hümitik asit uygulaması mandibula kırıklarının iyileşme zamanını kısaltabilir mi? Deneysel çalışma

**Amaç:** Bu pilot çalışmanın amacı tek bir lokal hümitik asit dozunun sıçanlarda subkondiler mandibula kırıkları üzerine etkilerini değerlendirmektir.

**Yöntem:** Bu çalışmada randomize deneysel protokol kullanıldı. Çalışma 16–18 haftalık 16 erkek Wistar albino sıçanlarıyla yürütüldü. Sıçanlar iki gruba randomize edildi. HA grubuna hümitik asit (0.3 cc/bölge, n=8) uygulanırken C grubuna (kontrol grubu, n=8) herhangi bir ilave ilaç verilmedi. Subkondiler alanda tam kalınlıkta cerrahi osteotomi yapıldı. Kırık hattının kemik yüzeylerine lokal olarak tek doz hümitik asit (0.3 cc/bölge) püskürtüldü. Postoperatif 21. gün mandibula diseke edildi. Daha sonra mandibulalar iki yarıya bölünerek histopatolojik inceleme yapıldı.

**Bulgular:** Kemik kırığı ortanca iyileşme skorları HA grubunda 7.16 (aralık: 7–8) ve C grubunda 7.50 (aralık: 7–8) idi. Gruplar iyileşme skorları açısından karşılaştırıldığında HA ile C grubu arasında istatistiksel açıdan herhangi bir anlamlı farklılık yoktu (p>0.05).

**Sonuç:** Bu çalışmanın sonuçları lokal hümitik asit uygulamasının kemik kırıklarının iyileşmesinde etkili olmadığını göstermiştir. Ancak, hümitik asidin farklı konsantrasyonları ve uygulama yöntemleri dahil, hem histopatolojik olarak hem de enflamatuvar ve proenflamatuvar sitokin düzeyleri açısından hümitik asidin etkilerini değerlendiren daha kapsamlı çalışmaların yürütülmesi gerektiği düşüncesindeyiz.

**Anahtar sözcükler:** Hümitik asit, subkondiler mandibüler kırıklar, kırık iyileşmesi.

Fracture healing is an exceptional biological course described by three intersecting phases: inflammatory reaction, callus arrangement, and bone remodeling.<sup>[1]</sup> Bone

regeneration is influenced by growth factors, cytokines and molecular signaling.<sup>[2–4]</sup> Numerous factors are included in regeneration and healing of the bone fracture. As

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per, TGF- $\beta$  is in charge for of mesenchymal stem cell stimulation, engaging osteoprogenitors, osteoblast and chondrocyte differentiation, chemotaxis, and bone matrix production.<sup>[5]</sup> Proinflammatory cytokines, including interleukin-1 (IL-1), IFN- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibit osteoblastic collagen production and interrupt formation of the bone matrix.<sup>[6,7]</sup> These cytokines also increase osteoclastic bone resorption.<sup>[7,8]</sup> As a result, a clear correlation exists between advanced inflammation and developed resorption of the bone. Thus, immune system activation and inflammation persuaded after bone fracture undesirably affect bone regeneration.

Humic acids (HA) are a group of high-molecular-weight polymers that are primarily derived from the decomposition of dead plants. HA is a dark-brown, carbon-rich material that mostly exists in peat, soil, and well water.<sup>[9]</sup> HA has been demonstrated to exist in the gastrointestinal tract of humans and animals and could circulate in the blood.<sup>[10]</sup> HA contains some trace elements that are important for human's health.<sup>[11]</sup> HA has been used therapeutically for a very long time. Some of its health-related effects are still unclear. Some studies showed that HA causes stimulation of lymphocytes and has antiviral, anti-allergic, anti-ulcerogenic, anti-inflammatory, and antibacterial properties.<sup>[12-14]</sup> In their study, Çalışır et al. showed that the systemic effects of HA have strong anti-inflammatory and osteoblastic activity.<sup>[11]</sup>

Mandibular fractures are the most common facial fractures. These fractures can be treated by using a conservative technique or surgery.<sup>[15]</sup> The most appropriate approach for treatment of subcondylar mandibular fractures has still been a matter of debate today.<sup>[16]</sup> Regardless of the method selected, possibility of development of various complications is present. In recent years, there has been an increase in the number of studies conducted by using the combinations of various techniques along with the method chosen for treatment of these fractures to shorten recovery time and reduce complications. In the study conducted by Altuntaş et al., for this purpose, which is involved in the present study, it was shown that subcondylar mandibular fractures recovery time was shortened by extracorporeal shock wave therapy.<sup>[17]</sup>

The results obtained from study of Çalışır et al. indicated that HA in systemic administration could stimulate bone recovery by causing an increase at anti-inflammatory cytokine levels like IL-10 but a decrease at proinflammatory cytokine levels like IL-1 $\beta$ .<sup>[11]</sup> In our literature reviews, we also could not found a study revealing the

effect of local administration of humic acid on recovery of bone fractures.

We aimed to seek an answer for the question "Can HA show this effect when it is also administered to surfaces of fractures locally?" in the present study in order to shorten recovery time of subcondylar mandibular fractures. We hypothesize that administration of HA can function as a novel treatment in bone fracture healing and shorten recovery time of subcondylar mandibular fractures.

## Materials and Methods

### Experimental animals

The experiments were carried out according to the National Institute of Health (NIH) Guide for the care and use of Laboratory Animals (NIH Publications No. 80-23 Revised 1996). The protocol of the study was approved by the Institutional Review and Animal Ethics Use Committee of Faculty of Medicine of Cumhuriyet University (65202830-050.04.04-19; date: 19.02.2015), and the study was conducted according to accepted guidelines for the care and use of laboratory animals for research.

In this study, a randomized experimental protocol was used. The study was conducted with 16 male Wistar-albino rats that were 16–18 weeks old and had an average body weight of 230 $\pm$ 10 g. Wistar albino rats (n=16) were randomly divided into two groups: Group HA received humic acid (0.3 cc/site, n=8) and Group C received no additional medical administration (control group, n=8).

The rats were kept under standard laboratory conditions (12-h light/dark cycles, 24 $\pm$ 2 °C, 35–60% humidity). Because of the broken jaws, all the animals were fed only with soft food and water for the first 7 days of experiment. The animals began their normal diets (a standard laboratory diet and available drinking water) after the first week.

### Drug and chemicals

Humic acid was obtained from SigmaTau (Rome, Italy). The humic acid solution (0.3 cc/site) was administered locally by spraying on the bone surfaces of the fracture line.

### Operation procedure and the study protocol

The animals were anesthetized with intraperitoneal injections of ketamine (7.5 mg/kg) (Ketalar®, Pfizer, Istanbul, Turkey) and xylazine (6 mg/kg) IM (Rompun®, Bayer, Istanbul, Turkey). Each right buccal area of the rats was

shaved and prepared with an antiseptic solution (povidone iodine). Following an approximately 10-mm incision made along the inferior border of the mandible and division of the masseter muscle, a full-thickness surgical osteotomy was made by using mosquito forceps in the subcondylar area, which was confirmed by condyle fragment mobility. Hemostasis was observed both on the fracture line and connected soft tissues, and a single dose of humic acid (0.3 cc/site, n=8) was sprayed in the Group HA by administering an injection. Nothing was administered to the Group C.

The wound was not syringed and no debris was removed. Finally, the skin flap was replaced and sutured. Intramuscular penicillin injections were administered with all the rats for the first 3 days after procedure.

After postoperative 21 day, the animals were euthanized with the intraperitoneal injections of pentothal sodium (200 mg/kg). Mandible was dissected and all soft tissues were removed after sacrificing process. Then, fractured hemimandibles were obtained for histopathological examination.

### Histopathological examination

The histological analyses were performed by two (HO, ET) pathologists blind to the samples. All tissue examples were immediately fixed in 10% formalin. After the fixation, specimens were kept at 10% nitric acid, decalcification was completed in 4 days and the specimens were embedded in paraffin. The specimens were cut in the sagittal sections into 5- $\mu$ m thick sections, transferred to slides for conventional hematoxylin-eosin (H&E) staining and examined by light microscopy (Eclipse 80I; Nikon, Tokyo, Japan). Digital camera and auxiliary equipment (USB (H) EXT 1/0; Nikon, Tokyo, Japan) were used with microscope to obtain digital images of the sections. The amount of the ossification for each section was scored out of 10 as described by Huo and Troiano<sup>[18]</sup> (Table 1). The total score of the scale ranged from 1 point (fibrous tissue) to 10 points (mature bone).

**Table 1.** Histological scoring system for the evaluation of fracture healing.

Score	Histological findings of the fracture zone
1 point	Fibrous tissue
2 points	Mainly fibrous tissue and small amount of cartilage tissue
3 points	Equal amount of fibrous and cartilage tissues
4 points	Completely cartilage tissue
5 points	Mainly cartilage tissue and small amount of immature (woven) bone
6 points	Equal amount of cartilage tissue and immature bone
7 points	Significantly immature (woven) bone and small amount of cartilage
8 points	Completely immature (woven) bone
9 points	Immature bone and small amount of mature bone
10 points	Mature (lamellar) bone

### Statistical analysis

The data were analyzed by using Statistical Package of Social Science (SPSS Inc., Chicago, IL, USA) for Windows version 22.0. Sections of all specimens stained with hematoxylin-eosin were scored. Mean scores were calculated for both groups and the differences between the groups were statistically analyzed. The data were expressed as mean, median, and minimum-maximum values. Bone fracture healing scores were analyzed by using Mann-Whitney U test. Level of significance was set at  $p < 0.05$ .

### Results

Two rats in the Group HA and two rats in the Group C died three days after the surgery due to dehydration and nutritional problems. Therefore, histological examination was not accomplished on these rats. Finally, 6 rats in both groups were included in the present study. All rats well tolerated the administration and no significant weight loss was observed until the end of the experiment.

Table 2 shows the histological scores of all specimens stained with H&E in terms of bone fracture healing.

**Table 2.** Histological scores of fracture healing for both groups.

	Minimum	Maximum	Median	Mode	Mean	p-value (MW)
Group HA, (n=6)	7	8	7	7	7.17 $\pm$ 0.41	12.000
Group C, (n=6)	7	8	7.5	7*	7.5 $\pm$ 0.55	0.394

\*Because the number of samples who received 7 and 8 points was equal, the smaller one was chosen.

The median score of bone fracture healing was 7.16 (range: 7 to 8) in the Group HA and 7.50 (range: 7 to 8) in the Group C. When the groups were compared in terms of bone healing scores, there was no statistical difference between the Group HA and the Group C ( $p>0.05$ ).

## Discussion

This study was designed to investigate the effect of a single dose administration of HA on bone healing and the histopathological features of the new bone. The present study showed that the controls and the rats administered with a single dose of HA did not show any significant difference in terms of bone healing scores.

The number of studies examining the effects of HA on bone tissue is rather limited. It was determined in study of Tkachenko et al.<sup>[9]</sup> that even though a significant increase was observed in recovery and formation of osteocyte when HA was administered to experimentally formed fracture line for a week, it caused a decrease in osteogenesis when administration time was extended. On the contrary, in the study on Kel'ginbaev et al.,<sup>[20]</sup> HA was observed to have a positive effect on regeneration of bone tissue. Similarly, in their study, Çalışır et al.<sup>[11]</sup> also observed that daily oral administration of 80 and 150 mg/kg HA decreased the alveolar bone loss and increased osteoblastic activity. The effect of systemic HA administration on bone recovery was examined in all three studies; however, we could not find a study evaluating local administration. Therefore, this is the first study examining the effect of local HA administration on bone.

It is known that proinflammatory and anti-inflammatory cytokine levels have an effect on recovery of bone fractures.<sup>[7]</sup> HA are the most common forms of organic carbon found in nature.<sup>[9]</sup> They exhibit strong anti-inflammatory effects by inhibiting IL-1 $\beta$  and TNF- $\alpha$  secretion activated by leukocytes.<sup>[21]</sup> Çalışır et al.<sup>[11]</sup> determined in their study that HA caused an increase in anti-inflammatory cytokine (IL-10) level and on the other hand, a decrease in proinflammatory cytokine (IL-1 $\beta$ ) levels when different doses of HA (20, 80 and 150 mg/kg) were administered via gastric feeding for 15 days in experimental rat periodontitis model. In the light of these results, they pointed out that 80 mg/kg/day HA administration had strong anti-inflammatory characteristics. Depending on this finding, they drew attention that HA could increase anti-inflammatory cytokine level and also enhance osteogenesis by decreasing production of proinflammatory cytokine. The study of Çalışır et al.<sup>[11]</sup> was a significant reference point in terms of our study's hypothesis. However,

we could not observe a similar effect in local single dose administration of HA.

There are studies in the literature, which indicates topical and spray forms of HA and its derivatives may be effective in wound healing, psoriasis, dermatitis, and rheumatoid arthritis treatment.<sup>[22]</sup> Therefore, the results of this study showed that local administration of HA was not efficient for healing of bone fractures. However, we are of the opinion that it is required to conduct more comprehensive studies, including HA's different concentrations and administration manners, evaluating the effects of HA on tissue both histopathologically and in terms of inflammatory and proinflammatory cytokine levels.

## Conclusion

The aim of the present pilot study was to investigate the effects of local administration of HA on bone fracture healing in an experimental model of subcondylar mandibular fractures in rats.

Despite the fact that the results of the present study showed that single dose local administration of HA did not have a positive effect on recovery of subcondylar mandibular fractures, it should be kept in mind that there are studies in the literature indicating HA is effective on bone recovery in systemic administration. For this reason, with the locally repeated HA administrations or methods, which may provide longer contact of HA with fracture surface during recovery time, in future, we think that the efficiency of HA should be examined in more details.

**Conflict of Interest:** No conflicts declared.

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