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Original research

THE EFFECT OF HUMIC ACID ON BONE REGENERATION IN RATS

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ABSTRACT

Objectives: The objective of our study was to investigate the effect of the local humic acid application in addition to autogenous bone grafting on the premature new bone amount.

Materials and Methods: 12-week-old 24 Wistar rats were divided into 4 groups. Two parietal bone defects with 5-mm diameter were constituted in each animal. All defects in each group were treated with only autogenous bone graft (n=6), autogenous bone graft combined with 100 mg/kg (n=6) or 200 mg/kg humic acid (n=6). Defects in one group remained empty as control (n=6). The laboratory animals were sacrificed on the 28th day following the procedure. Morphological properties of all experimental defects were evaluated using micro-computed tomography (Micro- CT).

Results: The highest value among trabecular thickness (Tb.Th) and the ratio of the bone volume to the tissue volume (BV/ TV) was encountered in the autogenous graft group applied with 100mg/kg humic acid. The highest value in the bone volume (BV) variance was detected in the group to which only the autogenous graft was applied. A statistically significant difference was found between the control group and the only autogenous graft-applied group and the autogenous graft group applied with local 100mg/kg humic acid upon comparing the groups in pairs for bone surface area (BS) variance. BS/BV value was found higher in the autogenous graft group applied with local 200mg/kg humic acid than the autogenous group applied with local 100mg/kg humic acid.

Conclusions: Humic acid application in addition to autogenous bone grafting caused a decrease in the bone volume value. However, the positive effect of the humic acid application was observed in trabecular thickness and bone volume/tissue volume values, dosage increase negatively impacted the same. The dosage increase negatively affected the bone surface area value. No positive effect of the humic acid application to bone surface/bone volume value was observed. The statistical significance between the control group and the other groups is considered to be autogenous graft.

Keywords: Humic acid, autogenous bone graft, bone regeneration.

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INTRODUCTION

Bone regeneration has been an important task in the field of dentistry. Considering aesthetical and functional demands, most clinicians aimed to obtain rapid bone augmentation with high quality. Therefore researches focus on the additional treatment modalities potentially accelerating the bone healing. The most frequently used surgical method in defect repair treatment is the bone grafting procedures.[1,2] Accordingly, the ideal bone graft material should have osteoinduction, osteoconduction and osteogenesis features, reportedly.[3] Autogenous bone grafts are accepted as the gold standard as the sole bone graft material possessing all these features, although they have disadvantages such as their being obtained at a limited amount, the observance of long-term postoperative disconfort.[4] Schlegel *et al*.[5] in their animal study, they used autogenous and bovine bone grafts in the sinus elevation operation. As a result of the study, they reported that the resorption rate of autogenous bone graft was 40% after 6 months, while this rate was 15% in bovine bone graft. Simion *et al*.[6] in their long-term retrospective study, they reported that autogenous bone particles used in guided bone regeneration are more prone to resorption.

Humic materials are organic carbon forms that already exist in nature. They represent many organic materials formed of earth, lignite, peat, mineral waters and precipitates. Humic materials are divided into three molecular forms: fulvic acids, humic acids and humin. Humic acids and fulvic acids disslove in an alkaline environment, while humans represent the unsolved residue. One of the essential parts of humic substances is humic acids.[7]

The naturally occurring humic acids are brown-black polymeric organic acids exist on earth and water surface.[8] Peat is an organic type of earth formed as a result of the humification of dead marsh plants. Peat has been used in the medical field for hundreds of years The organic part of the peat includes humin at the ratio of 90%, humic and fulvic acid (over 40%), lignin, polysaccharides, lipids, pectins, hemicellulose and cellulose.[9] Humic acids increase the vascularisation and thus accelerate the regeneration. Peat has been used for mud therapy (balneotherapy) for many years.[10] It is also used as an anti-inflammatory agent due to its antiinflammatory properties.[11,12] In addition, due to its antiviral, profibrinolytic, anti-inflammatory, estrogenic, antibacterial, anticancer, diuretic, and immunostimulant characteristics, it has been utilized for a variety of medical applications.[13-17] However, despite widespread research in the field of medicine, the research on the use of HA in the field of dentistry is insufficient .Peat-origin humic acid was used in this study.

Our aim in this study was to evaluate the effect of local humic acid use on insufficient new bone formation due to early period resorption, which is the disadvantage of autogenous bone grafts.

MATERIAL AND METHOD

The approval of the Animal Testing Ethical Committee of Sivas Cumhuriyet University was obtained on 17.06.2020 with line no. 328. 12-week-old 24 Wistar Albino rats with a weighing of 230-250 g were used as subjects. The conditions such as overall wellness of the laboratory animals and performance of no study over them previously were considered. All rats were fed with water and a standard diet, observing 12 hours night/day, 21±1ºC temperature, and 40-60% humidity ratio. Rats were caged in metal cages for 10 days to prepare for their new living conditions before the study. The testing stage of our study was conducted in Sivas Cumhuriyet University School of Medicine Animal Laboratory. Micro-CT imaging was carried out in Erciyes University Scientific Research Laboratory.

Group Formation

The laboratory animals were divided into 4 groups, and each group was formed of 6 laboratory animals. Those were determined as the control group, only autogenous graft-applied group, autogenous graft group applied with 100 mg/kg humic acid and autogenous graft group applied with 200 mg/kg humic acid (Table 1).

Table 1: Group formation

Experimental and control groups	Number of animals per group	The number of repetitions	Total number of animals used
Control Group	6	1	6
Only Autogenous Graft-Applied Group	6	1	6
Autogenous Graft Group Applied With 100 mg/kg Humic Acid	6	1	6
Autogenous Graft Group Applied With 200 mg/kg Humic Acid	6	1	6

Surgical Method

Rats were subjected to anesthesia through 30 mg/kg Ketamin-HCL (Ketas, Eczacıbaşı, Turkey) and 5 mg/kg Xylazine HCL (Rompun, Bayer, Germany) injection, the skin on the parietal area of the laboratory animals were shaved, and the operation zone was wiped using povidin iodine and covered with a sterile surgical cover. Then cranial cutaneous incision was applied, extending from the occipital to the frontal bone for about 2 cm. All tissues were dissected in a manner to include skin, subcutaneous tissue and periost, respectively, in order to reveal the parietal bone. Automax drill was used to obtain a 5mm-diameter bicortical bone fragment at critical size after the bone surface appeared. After exposing the bone surface, a critically sized 5mm diameter bicortical bone fragment was removed. The defect was formed under psychological saline solution irrigation at 600-1000 rpm. Then, the bone tissue formed as a particulate autogenous graft, which was obtained during the formation, was placed in the defected zone of the relevant test group. The skin flap was sutured to its original position using 5-0 polyglactin 910 suture following the closure of the under-skin fascia after the operation.

Humic Acid Application

The suitable humic acid concentration was prepared for the rats to which local humic acid would be applied; the amount of humic acid determined for the groups of local application was applied by being mixed with the autogenous graft.

Termination of the Test

The laboratory animals were sacrificed using 200mg/kg sodium pentobarbital (Petothal, Abbot, USA) on the 28th day following the process. Then, the grafted defect zone was removed with sufficient bones around it and placed in 10% formalin.

Evaluation by Micro-Computerized Tomography (Micro-CT)

Micro-CT was first started in 1989 by Feldkamp *et al*.[18] Tissue mineral density, bone mineral density and bone volume can be evaluated in Micro-CT. It is also accepted as a golden standard for evaluating the three-dimensional structure of trabecular bone. However, it is not successful in the evaluation of the microstructure of cortical bone.[18,19] The Micro-CT (SkyScan-1272, Bruker, Kontich, Belgium) device belonging to Erciyes University Faculty of Dentistry Research Laboratory was used in the study.

Micro-CT helps to analyze the microstructure of the bone trabecules and performing BMD (bone mineral density) measurement. Furthermore BV (new bone volume), BV/TV, BS (new bone surface), BS/BV (new bone surface/BV), Tb.Th (trabecular thickness), Tb. N (trabecular number) values were examined in the area of examination.[20,21]

Statistical Method

The data obtained in our study was loaded to SPSS (Ver 22.0) software and was obtained through measurement as the parametric test assumptions were performed in the evaluation of the data (Kolmogorov-Smirnov), Variance Analysis was used to compare the measurements obtained from more than two independent groups, and Tukey test was used to find the differentiating group and groups when the difference between groups was found significant at the end of the analysis. The level of significance was accepted as $p<0.05$.

RESULTS

Micro-CT analysis results

The biopsy samples were subjected to radiologic examination in the micro-CT device. The result of the analysis is described in table 2.

Table 2: Intergroup comparison of micro-CT mean values

Variables	Control group	Only autogenous graft-applied group	Autogenous graft group applied with 100 mg/kg humic acid	Autogenous graft group applied with 200 mg/kg humic acid
BV(mm^3)	3.23±1.31[a]	8.91±2,47[b]	7.33±1,88[b]	6.70±2.11[b]
BV/TV(%)	12.20±5.77[a]	25.51±4.75[ab]	28.88±4.44[b]	20.69±6.77[ab]
BS(mm^2)	41.67±13.09[a]	85.77±18.39[b]	69.14±13.33[b]	64.67±18.04[ab]
BS/BV(%)	13.48±2.49[a]	9.76±1.10[b]	9.26±0.76[b]	9.82±1.19[b]
Tb.Th(mm)	0.29±0.06[a]	0.39±0.02[b]	0.41±0.02[b]	0.40±0.03[b]
Tb.N(1/mm)	0.41 ±0.16	0.63± 0.10	0.53± 0.22	0.50± 0.13

Data are expressed as mean ± standard deviation. Similar letters on the same line indicate the similarity between groups, and different letters indicate the difference between groups.

The highest value in BV variance was only detected in the autogenous graft-applied group (8.91±2.47mm^3), and the lowest was observed in the control group (3.23±1.31mm^3). Comparing the groups in pairs, the difference between the control group and the other groups was statistically significant (p<0.05). Likewise, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BV value in the autogenous graft group applied with local 100mg/kg humic acid was found higher.

Considering the BS/BV rates, while the highest value was observed in the control group (13.48±2.49%), the lowest was detected in the autogenous graft group applied with local 100mg/kg humic acid (9.26±0.76%). In pairwise comparison of the groups, a statistically significant difference was found between the control group and the other groups (p<0.05). Similarly, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BS/ BV rate in the autogenous graft group applied with local 200mg/kg humic acid was found higher.

The highest value was found in the local 100mg/kg humic acid-applied autogenous graft group (0.41±0.02mm), and the lowest was found in the control group (0.29±0.06mm) in Tb.The variance. Comparing the groups in pairs, a statistically significant difference was found between the control group and the other groups (p<0.05). In a similar manner, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the Tb. The value in the autogenous graft group applied with local 100mg/kg humic acid was found higher.

The highest value for BV/TV variance was detected in the local 100mg/kg humic acid-applied autogenous graft group (28.88±4.44%), and the lowest value was found in the control group (12.20±5.77%). Upon comparing the groups in pairs, When the groups are compared in pairs, a statistically significant difference was found between the control group and the local 100mg/kg humic acid-applied autogenous graft group (p<0.05). At the same time, also the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BV/TV value in the autogenous graft group applied with local 100mg/kg humic acid was found higher.

Upon comprising the groups in pairs for BS variance, a statistically significant difference was found between the control group and the only autogenous graft-applied group and local 100mg/kg humic acid applied autogenous graft group (p<0.05), the difference between the local 200mg/kg humic acid-applied autogenous graft group was not found statistically significant (p>0.05). At the same time, Furthermore the difference between the autogenous graft group applied with local 100mg/kg humic acid and the

autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BS value in the autogenous graft group applied with local 100mg/kg humic acid was found higher.

Upon comparing in comparison, Tb/N (1/mm) variance groups, although no statistically significant difference was found among groups, the highest value was found in the only autogenous graft-applied group, and the lowest was found in the control group.

DISCUSSION

The autogenous graft can be obtained from intraoral zones such as tuber maxilla, mandibular ramus, mandibular symphysis and the toothless zone or extraoral zones such as iliac bone, tibia and skull.[22] Although autogenous grafts are presented as the golden standard, their clinical activities are limited due to the scarcity of the obtained graft amount and resorption.[23] Given all these advantages and disadvantages, the autogenous graft has been the scope of our study similar to many other studies.[18,24]

Humic acids are the most widely available organic carbon forms, and they exhibit strong anti-inflammatory effects by inhibiting IL-1β ve TNF-α release, which is activated by leucocytes.[25] It was reported that humic acid significantly degrades liposaccharide-mediated adhesion molecules (ICAM-1, VCAM-1 and E-selectin) being cultured from human umbilical and endothelial cells.[26] This can be deemed among the methods to describe the possible effects of humic acid on the inflammatory process. In a study in which individuals suffering from HIV were treated using 2, 4, 6 and 8 grams of oxymate/day for 2 weeks, no sign of toxicity was encountered at the end of the period.[27]

In the study conducted by Van Rensburg *et al.*[25], the effects of potassium humins on TNF-α, IL-1β, IL-6 and IL-10 synthesis were obtained at a 40μg / ml concentration. In this concentration, it was reported that humins significantly inhibited TNF-α, IL-1β, IL-6 and IL-10 release produced by phytohemagglutinin (PHA), which is stimulated by mononuclear leukocytes (MNL).

It was reported that the oral use of the humin (leonardite) for 6 days, which is obtained from daily 61 mg /kg lignite compressed contact hypersensitivity in rats. Furthermore, no toxicity was observed as a result of the use of 1000 mg /kg leonardite human orally for 1 month in rats. The teratogenic effect was not observed as a result of the treatment of pregnant rats with 500 mg /kg humans between the 5th and 17th days of their pregnancy.[24]

Humic acid acts as a dilator increasing the cell wall permeability. This increasing permeability eases mineral transfer from blood to bone and cells. Furthermore, it was reported that humic acid increases the calcification of xenografts by 16%.[28]

We researched the effect of humic acid on the healing mechanism of bone when it is applied locally in the light of this information.

It was reported that the regenerative capacity of cranial defects in laboratory animal models was better than in human beings. Calvarium develops morphologically and embryologically from a membrane process and resembles other bones developing in a membranous way on the face. As calvarium has two cortical layers anatomically, it resembles a structure like a mandibula and the physiological structure of atrophic mandibula. Due to such reasons, calvaria is one of the most preferred test zones.[29,30] We preferred it due to reasons such as the simplicity of the operational intervention to the parietal bone, the low possibility of harming the relevant part of the animal following the operation, osteogenic potential of dura mater and periost. Besides these advantages, the fact that the bone is about 2 mm thick in the parietal zone, bleeding depending on the dura mater perforation during operation and brain damage constitutes the risk factors requiring us to be sensitive in our study.

Critical size defect is a size defect with no chance to heal itself both in terms of function and form as long as the bone tissue is alive. When the critical defect size is exceeded, healing occurs through connective tissue, not through bone tissue. The critical size of bone tissue wound is dependent on factors such as the type of the

defect, age, gender, zone, depth and systemic condition.[31-33]

Takagi and Urist[34], examined the bone defect of 8 mm in diameter, which they formed in rats after waiting for 6 months and reported that fibrous healing occurred at the end of this period. In another study, Mulliken and Glowacki[35,36], reported that a 2-mm defect on the parietal bone in rats is not an ideal for ossification after 6 months. As in these studies, defects with various diameters were formed, and different results were stated. Please take note that there are different factors together with defect diameter concerning the difference in results. It was reported that causes such as trauma, insufficient blood support, bone marrow deficiency, the infection might affect the result.[32,37]

As it is reported in the studies, it has been understood that there was no consensus about the critical defect size. In many studies conducted, it was reported that a defect of 5 mm in diameter was sufficient for a critical size defect.[38,39] In the light of this information, it was decided to take the defect size as 5 mm in diameter in our study.

Different sacrification times were expected upon examining bone healing in the studies conducted. Generally, sacrification occurred at 2-8 week intervals. Sacrification was performed in the 4th week in order to track premature bone formation in our study.[33]

Micro-CT examinations are also used in human studies. However, these practices mandates conducting biopsy on bone. It is a much more frequently used imaging method in animal studies. Histomorphometric and micro-CT evaluations give quite objective results by providing digital data in the micromorphological examinations of bone. Micro-CT imaging provides fast, simple and much more precise measurements compared to histomorphometric evaluations. At the same time, histomorphometric evaluations provide only 2-dimensional sections; however, micro-CT evaluations enable volumetric measurements through 3-dimensional images.[20,40] Micro-CT imaging method providing digital values through 3-dimensional volumetric measurements was used in our study.

There are many radiographically examined micro-CT evaluation studies related to bone healing and micromorphology in the literature.[41-43] No study evaluating the effect of humic acid on bone regeneration in bone defects formed in rats through Micro-CT examination is available in the literature. Our study may contribute to the literature in terms of giving opinions to the relevant dentists concerning the effect of the local humic acid application on bone regeneration in bone defects from this aspect.

Çalışır *et al*.[44] stated that the systemic effects of humic acid have potent anti-inflammatory and osteoblastic activity, may stimulate bone healing by causing an increase in the anti-inflammatory cytokine levels such as IL-10 however a decrease in proinflammatory cytokine levels such as IL-1β. Furthermore, they observed that daily oral 80 and 150 mg/kg humic acid application decreases alveolar bone loss and increases osteoblastic activity.

Tkachenko *et al.*[45] determined in their study that although a significant increase was observed in healing and formation of osteocytes when applied for one week on the fracture line formed experimentally, it caused a decrease in osteogenesis when application time was extended.

In the study conducted by Kel'ginbaev *et al.*[46] it was seen that humic acid had a positive effect on the regeneration of bone tissue.

Durmuş *et al*.[47] used 16 Wister Albino rats in the study in which they research local humic acid application to healing time in mandibula fractures. Rats were grouped as control group and local humic acid (0.3cc/zone) group. They applied a single dose of humic acid (0.3cc/region) locally to the bone surfaces of the fracture line by raising a full thickness flap in the subcondylar region and performing a surgical osteotomy. They reported that humic acid was not effective in the healing of bone fractures in local single dose application.

In our study, the highest value in the BV variable was found only in the autogenous graft

group (8.91±2.47mm3). At the same time, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BV value in the autogenous graft group applied with local 100mg/kg humic acid was found higher. The highest value was found in Tb in the local 100mg/kg humic acid-applied autogenous graft group (0.41±0.02mm). The variance. At the same time, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the Tb.Th value in the autogenous graft group applied with local 100mg/kg humic acid was found higher. The highest BV/TV varisance value was detected in the local 100mg/kg humic acid-applied autogenous graft group (28.88±4.44%). At the same time, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BV/ TV value in the autogenous graft group applied with local 100mg/kg humic acid was found higher. At the same time, the difference between the autogenous graft group applied with local 100mg/kg humic acid and the autogenous graft group applied with local 200mg/kg humic acid was not statistically significant; however, the BS value in the autogenous graft group applied with local 100mg/kg humic acid was found higher. In the light of these findings, it can be considered that local humic acid application increased bone regeneration at a limited level. However, a positive effect of increasing the locally applied humic acid ratio on bone regeneration was not found. More studies should be conducted regarding this matter.

CONCLUSIONS

According to Micro-CT results:

- ❖ Humic acid application in addition to autogenous bone graftcaused a decrease in BV value.
- ❖ Although the positive effect of the humic acid application was observed in Tb.Th and BV/TV values, dosage increase negatively impacted the same.
- ❖ The dosage increase also negatively affected the BS value.
- ❖ No positive effect of the humic acid application to BS/BV value was observed.
- ❖ The reason behind the statistical significance between the control group and the test groups is attributed to high regenerative potential of autogenous graft, rather that humic acid.

Considering the limitations of animal study models, it is considered that humic acid may have a limited positive effect when applied in doses suitable to ossification.

This is the first study evaluating additional benefit of humic acid in bone regeneration in combination with autogenous bone grafts. We think that the results that we obtained from this study will contribute to the literature on the clinical use of humic acid based on the results that we obtained.

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DECLARATIONS OF INTEREST

"No potential conflict of interest was reported by the authors."

Humik Asitin Kemik Rejenerasyonu Üzerine Etkisinin Araştırılması

ÖZ

Amaç: *Çalışmamızın amacı, otojen kemik grefti uygulamalarında lokal humik asitin erken dönem oluşan yeni kemik miktarı üzerine etkisinin araştırılmasıdır.* ***Gereç ve yöntemler:*** *Denek olarak 12 haftalık, ortalama ağırlıkları 230-250 g olan Wistar Albino cinsi 24 adet rat kullanılmıştır. Deney hayvanları 4 gruba ayrılmıştır. Bunlar kontrol grubu,*

sadece otojen greft uygulanan grup, lokal 100 mg/kg humik asitle uygulanan otojen greft grubu ve lokal 200 mg/kg humik asitle uygulanan otojen greft grubu şeklinde belirlenmiştir. Deney hayvanlarının parietal kemiğinde kritik boyutta 5mm çapında bikortikal kemik fragmanı automax frez kullanarak serum fizyolojik irrigasyonu altında çıkarılmıştır. Defektli bölgeye oluşturulma esnasında elde edilen otojen greft ilgili deney gruplarına uygulanmıştır. Lokal uygulama yapılacak gruplar için belirlenen humik asit miktarı otojen greftle karıştırılarak uygulanmıştır. Deney hayvanları işlem sonrası 28.günde sakrifiye edilmiştir. Daha sonra greftlenen defekt bölgesi mikro-bilgisayarlı tomografi (Mikro- BT) ile değerlendirilmiştir. ***Bulgular:*** *Trabeküler kalınlık (Tb.Th) ve kemik hacminin doku hacmine oranı (BV/ TV) değerlerinde en yüksek değer lokal 100mg/kg humik asitle uygulanan otojen greft grubunda bulunmuştur. Kemik hacmi (BV) değişkeninde en yüksek değer sadece otojen greft uygulanan grupta tespit edilmiştir. Kemik yüzey alanı (BS) değişkeni için gruplar ikişerli olarak karşılaştırıldığında kontrol grubuyla sadece otojen greft uygulanan grup ve lokal 100mg/kg humik asitle uygulanan otojen greft grubu arasında istatistiksel olarak anlamlı fark bulunmuştur. Lokal 100mg/kg humik asitle uygulanan otojen greft gruba göre lokal 200mg/kg humik asitle uygulanan otojen greft grubunda BS/BV değeri daha yüksek bulunmuştur.* ***Sonuçlar:*** *Humik asit uygulandığında kemik hacmi değerinde azalmaya sebep olmuştur. trabeküler kalınlık ve kemik hcmi/doku hacmi değerlerinde humik asit uygulaması olumlu etkisi görülmesine rağmen doz artırılması olumsuz etkilemiştir. Kemik yüzey alanı değerinde de doz artırılması olumsuz etkilemiştir. Kemik yüzey alanı/ kemik hacmi değerine humik asit uygulamasıın olumlu etkisi görülmemiştir. Kontrol grubuyla diğer gruplar arsındaki istatistiksel olarak anlamlı çıkmasının nedeni otojen greft olduğu düşünülmektedir.* ***Anahtar kelimeler:*** *Humik asit, otojen kemik grefti, kemik rejenerasyonu.*

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