Original Article

Comparison of Efficiency of Hyaluronic Acid and/or Bone Grafts in Healing of Bone Defects

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Background: Reconstruction of bone defects in oral and maxillofacial surgery has widespread uses. In recent years, the capacity of various biomaterials alone or in combination with bone graft materials to increase bone healing has been an intensive research topic. The aim of this study is to evaluate the efficacy of hyaluronic acid and/or bone graft material on bone healing in defects created in the rat mandible. Methods: In our study, rats were divided into 4 groups. Group 1 is designated to be treated with no materials, Group 2 with graft material, Group 3 with only hyaluronic acid, and Group 4 with hyaluronic acid and graft material. A critical-size defect of 5 mm in diameter was created bilaterally in the rat mandibles and the rats were divided into the indicated groups accordingly. At the end of the postoperative 6th week, the experiment was terminated. The right halves of the mandibles were evaluated immunohistochemically and histopathologically in terms of bone healing, and the left in terms of mineralization level via microcomputed tomography. Results: Histopathological evaluation showed that healing in the empty group was significantly lower than the other groups that were treated with materials (P < 0.05); but the difference between the material-treated groups was not significant. Immunohistochemical evaluation revealed that the staining was moderately positive/strongly positive in all groups, but the difference between the groups was not significant. The highest mineralization values observed in the defected areas that belonged to 2 groups using hyaluronic acid, and the difference between them was found to be statistically significant (P < 0.05). The lowest mineralization values observed in the defected areas was most frequent in the group where only the hyaluronic acid was used, and there was a statistically significant difference between the other groups (P < 0.05). Conclusion: In conclusion, the use of hyaluronic acid alone or in combination with bone grafting has been shown to contribute positively to the improvement of bone defects in the jaw area.

Keywords: Bone defect, hyaluronic acid, mandible, rat

INTRODUCTION

Bone tissue is a well-organized and special tissue hardened by the calcium buildup in its composition. Bone tissue, which renews itself through life-long remodeling and can self-repair, is the only tissue that can heal without scarring. However, bone tissue fails to self-heal the defect in defects over a certain size. Such defects are called critical-size defects and various biomaterials or bone grafts are used in their reconstruction. The main task of bone grafts or biomaterials used in reconstruction of bone defects is to ensure that the defects are healed as quickly as possible and anatomically consistent, and to guide the newly formed tissues. An ideal bone graft/biomaterial should be resistant to mechanical forces on the surgery

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region, should be available in sufficient quantity, should be cost-effective, should not require a second operation, can easily be shaped into desired form, should be easy to apply, can be stored for a long time, and should not carry an antigenic property. The materials we use today can only meet some of these properties.

Another biomaterial that has recently begun to be used in oral and maxillofacial surgery is hyaluronic acid (HA). HA is a member of the glycosaminoglycan family and is an important component of the extracellular matrix. This material is synthesized by almost every cell in the body. In addition to its role in preserving and supporting the structural integrity of extracellular matrix, HA, with its high-water binding capacity, also performs tasks such as balancing the osmotic pressure and providing cell-cell and cell-matrix interactions as its main tasks. Besides, it also has bacteriostatic property. In addition to these basic properties, it can also assume different tasks depending on the nature of the tissue, type of cell it is secreted by, and its molecular weight.

HA has important task in bone tissue as well as in other tissues. It interacts with cells involved in bone metabolism (monocyte, fibroblast, osteoblast, osteoclast, osteocyte), as well as proteins (growth factors, collagen type I, collagen type V, fibronectin, calcitonin). It helps osteoblast, osteoclast, and osteocyte cells, each of which plays a role in bone formation, fulfilling functions such as migration, differentiation, and proliferation.

Usage of this biomaterial, which is frequently preferred in the fields of ophthalmology, orthopedics, and dermatology because of its above-mentioned multifunctional structure, in oral and maxillofacial surgery is a novel application.

As mentioned, we think that HA, a vital molecule for the cell and its environment, should be considered in the search for a more effective biomaterial for bone healing. The aim of our study is to assess the efficacy of HA on bone healing on the 6th week after it is applied alone and/or in combination with bone graft on the critical-size defect area formed on rat mandible immunohistochemically/histopathologically, and the mineralization level of the newly formed tissue via microcomputed tomography.

**Materials and Method**

**Experimental animals**

In our study, 40 12-month-old Sprague-Dawley male rats weighing between 450-550 g that completed their skeletal development were used as experimental animals. The local ethics committee for animal ethics evaluated and approved our study.

**Biomaterials used**

In our study, particle form of freeze-dried human cortical bone was used as bone graft (DuraGraft, Gainesville, United States); as for the HA, the form of the same bone obtained by bacterial fermentation (Hyaloss, Reggio Emilia, Italy) was used. The HA used in our study was in fibrous form and produced by partial esterification of propiophenone derivatives of the carboxylic groups with the hydroxyl groups.

**Surgical method**

General anesthesia was achieved by intraperitoneally injecting 80 mg/kg Ketamine HCl (Alfamine®, Ege-Vet, Turkey) and 12 mg/kg Xylazine HCl2 (Alfazyne, Ege-Vet, Turkey) to each experimental animal. The skin on both right and left sides of the operation region was shaved after it had been wiped with iodine solution. After the heads of the rats were positioned on the foam block, they were fixed on it by the skin in the nape region with the aid of a 10 cc injector. The incision was made longitudinally at the skin level, approximately 3 mm above and parallel to the lower edge of the mandible. The bone tissue was reached via blunt dissection of the muscles and sharp dissection of the periosteum. With the help of the small Langenbeck retractor, the muscles were excised superiorly and inferiorly; the lateral surface of the ramus had been clearly revealed in a way that the posterior and inferior margin of the mandible and sigmoid notch were clearly visible. The area where the defect was to be surgically created was marked with a pencil 3 mm above the lower edge of the mandible, 2 mm forward from the posterior edge of the mandible, and 2 mm below the sigmoid notch anatomic structure. Bicortical osteotomy was performed under physiological saline irrigation with a 5-mm round-tipped steel surgical trephine bur to avoid exceeding these boundaries, and a well-defined defect was created [Figure 1].

**Biomaterial application**

The experimental animals were divided into 4 groups with 10 subjects in each group. The defect area was left empty for the animals in group 1. For the animals in group 2, the defect area was completely filled with particulate graft (DuraGraft, Gainesville, United States) [Figure 2]. For the animals in group 3, a half of track of HA was cut and placed in the defect area [Figure 3]. In group 4, HA was placed to fill the half of a tract, and then the remaining space was filled with particulate graft.

Muscle and skin tissues were separately sutured with 4/0 vicryl polyglactin (Dogsan, Trabzon, Turkey) with continuous locking suture technique. All operations were performed for the other region of the mandible.
following the same procedure. Surgical procedures of all experimental animals were performed by one person.

Postoperative follow-up
On the day of the surgical operation and the following 2 days, all rats were given intramuscular 10 mg/kg/day, amikacin sulphate (AmikosisÔ, Eczacıbaşı, Turkey) for infection control, and for pain and antibiotics control, rats were analgesically administered intraperitoneal 5 mg/kg/day, ketoprofen (ProfenidÔ ampoule, Senofı Aventis, Turkey).

In the postoperative period, experimental animals were given normal tap water and fed with special diets containing 21% protein. During the course of the experiment, rats were kept in optimized small animal care rooms with 21-22°C room temperature and 50% humidity in single-masted cages, and their general state of health, behavior patterns, feed and water intake were monitored.

Obtaining samples
At the end of the postoperative 6th week, the animals were sacrificed by doubling the dose of anesthesia. The mandible was removed as one piece and separated into two separate pieces from the symphysis area after the surrounding soft tissues and muscles are dissected. Each part of the mandible was stored in 10% formalin (Merck, Darmstadt, Germany).

Samples taken from the right half of the mandible were analyzed histopathologically and immunohistochemically for evaluation of bone healing. The samples taken from the left half of the mandible were evaluated via microcomputed tomography in terms of bone density. Samples had been coded before they were sent to the relevant laboratories for examination purposes, and information about which group the samples belonged to was not shared with the people who carry out the evaluation.

Histopathological and immunohistochemical examination
Histopathologic and immunohistochemical examinations were reviewed twice by the same pathologist at different times. Samples had been left in decalcification solution (5% nitric acid) for 2 weeks before examinations were performed. The softening samples were divided into three separate parts from the defect area. This way, the defect area was examined at three different levels.

Histopathological examination
Bone samples of each rat were placed on separate follow-up trays. After the tissue had been washed under the stream for 1 h, it was placed in the tissue processing device. Throughout the night, the tissues were run through low-grade alcohols to high-grade alcohols (from 70% to 100%) to remove the water from the contents. Following this process, they were run through xylol two times to purify the oil in the contents. Finally, they were run through warm paraffin to fill the tissue cavities with paraffin. The next day, tissues were embedded in paraffin for blocking procedure. After the tissues had been cooled for 4-5 h in the blocks, sections 5 micron in thickness were taken at two different levels with a rotary microtome. This way, the three-part defected area was sampled as six different areas with its sections at two different levels.

After the sections had been stained with hematoxylin eosin, they were examined under a light microscope with X400 magnification. Bone healing at the defect area as scored using the Emery scale, the lowest value of histopathological healing was specified as 0 and the highest value as 7 as described in Table 1.

Immunohistochemical examination
In the immunoperoxidase method used for immunohistochemical examination, the streptavidin-biotin peroxidase method was preferred. Osteopontin, osterix, and alkaline phosphatase activities on the newly formed tissue were examined with anti-osteopontin antibody (ab8448, 1/100 dilution), anti-Sp7/osterix antibody-ChIP Grade (ab22552, 1/100 dilution) and anti-alkaline phosphatase, tissue nonspecific antibody (ab65834, 1/100 dilution) kits (Abcam, Cambridge, United Kingdom).

The sections were run through graded alcohols and xylol respectively and following these procedures, they were dehydrated and deparaffinized. The tissues were washed in water for 10 min and treated with hydrogen peroxide in 3% methanol for 20 min to eliminate the endogenous peroxidase activity they contained. Following this procedure, the tissues were washed twice in phosphate buffer saline solution for 10 min each wash. After that they were kept in normal serum for 45 min to prevent nonspecific staining. After this step, the primer sera were applied without washing and the samples are stored at + 4°C for 1 night. The following day, the tissues were washed in PBS in the same fashion and for the same duration, after which they were treated with biotinylated serum for 30 min and washed again in phosphate buffer saline twice for 10 min each wash. After this procedure, the tissues were treated with streptavidin alkaline phosphatase conjugate for 30 min and washed again with phosphate buffer saline. Then, they were incubated with biotinylated secondary antibody for 30 min. Following these procedures, the samples were stained with 3,3 diaminobenzidine chromogen. Harris hematoxylin was used for counter staining and the preparations were examined under light microscope.
Densities of osteopontin, osterix, and alkaline phosphatase marked in each group were measured according to the classification between a scale of 0-3 [Table 2].

The stained sections were photographed using an Olympus CX41 model microscope using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

**Microcomputed tomography evaluation**

A microcomputed tomography device (SkyScan 1174 compact Micro CT, Kontich, Belgium) was used to evaluate the density of newly formed bone tissue. In our study, bone samples were examined twice by the same person at different times. Bone samples from each rat were dried with a paper towel to remove the formaldehyde solution they contain. Slightly moistened bone samples were placed perpendicular to the exact center of the 6 cm diameter cylindrical molds. The samples were stabilized in the molds with the aid of a transparent acrylic resin (Orthoplast, Vertex, Zest, The Netherlands). The samples fixed in the cylindrical molds for analysis were then placed on the sample table inside the microcomputed tomography device.

The Flat-Field software was used to scan each sample. In this software, the scanning time was set to 2300 ms and the rotation speed was set to 0.7°. All scans were done at a 180° angle of rotation. During this time, three images were obtained at every 0.7° rotation. After the scanning process was completed, 33 mm thick transverse sections were taken from the raw data obtained with the NRecon software. A total of 272 sections were obtained for each sample. The obtained sections were converted to bmp format and transferred to CTan software.

After the sections were transferred to the software, the relevant areas were selected in the defect area with the region of interest. According to the images obtained with region of interest from the sections, the lower limit of the defect was accepted as the 0 point and the area at 5 mm distance to this point was marked. The different mineralization levels in bone islands that had been newly formed between these two points were evaluated in 3-D and the areas with different densities were recorded as “Gray Areas.” The area with the lowest mineralization was named the low gray area (LGA) and the area with the highest mineralization was named high gray area (HGA). This protocol, which allows for the evaluation of the level of mineralization in the newly formed bone tissue, was followed in the same way for each bone sample.

**Statistical analysis**

Statistical analysis of the data was performed using SPSS (Statistical Package for Social Sciences, Chicago IL, USA) 20.0 packaged software. Whether the obtained data had met the requirement of compliance with normal distribution, one of the preconditions of parametric tests, was analyzed by applying the Anderson-Darling test. Whether the precondition of the homogeneity of variance covariance matrix, one of the most important conditions of the parametric tests, had been met was examined by applying the Box’s M test. Since the new bone formation scores, alkaline phosphatase, osteopontin, osterix markers density scores, and LGA-HGA values did not satisfy the variance homogeneity of parametric tests and the normal values of observations, cross-group comparisons were analyzed by applying Kruskall-Wallis tests. Multiple Bonferroni-Dunn tests were applied for these characteristics to determine the differences between the groups.

**Results**

**Distribution of samples by group**

Out of total 40 experimental animals included in the study, 8 of them were eliminated from the study because of the development of mandibular fracture during operation, 2 were eliminated because of abscess formation in defect area, 3 were eliminated because of occurrence of death before the completion of the study. Also, because of the observed fractures on the right half of the mandible of 2 subjects, and on the left half of the mandible of 1 subject, the related samples were not included in the study.

Although a total of 27 bone samples were examined histopathologically and immunohistochemically in the study, 28 bone samples were evaluated in terms of bone density.

**Histopathological bone healing**

Histopathological examinations revealed proliferation of bone tissue at the defect borders in all groups in the postoperative 6th week. Intense

![Figure 1: 5 mm diameter defect formed via trephine drill](image-url)
Figure 2: Particle bone placed on the defect region

Figure 3: HA placed on the defect region

Figure 4: An example of bone healing from Group 1. Arrowheads indicate bone tissue around the defect, arrows indicate the newly formed tissue (Bar = 100 μm)

Figure 5: An example of bone healing from Group 2. Arrowheads indicate bone tissue around the defect, black arrows indicate inflammatory cell infiltration, white arrows indicate the newly developed bone tissue (Bar = 100 μm)

Figure 6: An example of bone healing from Group 3. Arrowheads indicate bone tissue around the defect, black arrows indicate inflammatory cell infiltration, white arrows indicate the newly developed bone tissue (Bar = 100 μm)

Figure 7: An example of bone healing from Group 4. Arrowheads indicate bone tissue around the defect, black arrows indicate inflammatory cell infiltration, white arrows indicate the newly developed bone tissue (Bar = 100 μm)
The difference between the bone healing values of Group 1 and the values of Group 2, Group 3, and Group 4 were found to be statistically significant ($P < 0.01$ for all groups). Although the scale average of bone healing belonging to the group where grafts and HA had been used was higher than the other two groups where biomaterials had been used, the difference between them was not statistically significant. The average bone healing score was found to be $4.67 \pm 0.41$ in Group 2, $4.67 \pm 0.41$ in Group 3, and $5.60 \pm 0.25$ in Group 4.

**Activity of bone formation markers**

An increase in the activity was observed in all of the examined markers. Although the increase in the activity of these markers in the connective tissue and inflammatory cells was more severe, the increase was observed to be milder in bone and muscle tissue.

According to the scoring, where the lowest value is 0 and the highest value is 3, used in the measurement of the activity of bone formation markers alkaline phosphatase, osteopontin, and osterix, the activity of each of the 3 bone markers studied was usually 2-3 (moderate to severe positive). There was no statistically significant difference in the level of bone markers between the groups studied.

**Bone density**

LGA is the area where the level of bone mineralization was observed to be the lowest; HGA is the area where bone mineralization level was observed to be the highest in the newly ossified tissue. In the samples examined in our study, while the LGA areas were seen in the center or near the center of the defect area, HGA areas were seen at the borders of the defect area or near the borders.

The samples from Group 1 ($57.60 \pm 1.60$) had the lowest mean LGA, whereas the samples from Group 3 ($69.33 \pm 3.98$) had the highest mean LGA. The LGA values of both groups were statistically different from the other groups ($P < 0.05$ for both groups). There was no statistically significant difference between Group 2 ($63.80 \pm 0.20$) and Group 4 ($64.25 \pm 3.33$) [Table 3].

Group 3 ($242.56 \pm 2.80$) and Group 4 ($241.3 \pm 13.8$) were the groups with the highest mean HGA values. Group 1 ($221.80 \pm 5.20$) and Group 2 ($227.00 \pm 0.00$) on the other hand were the groups with the lowest mean HGA values. The difference between the mean values of Group 3 and Group 4 and the mean values of Group 1 and Group 2 was found to be statistically significant ($P < 0.05$). However, there was no significant difference in terms of HGA values between samples belonging to Group 1 and Group 2 and samples belonging to Group 3 and Group 4 [Table 4].
**DISCUSSION**

Bone grafts and biomaterials have a wide range of uses in the fields of oral and maxillofacial surgery. Thanks to its diverse functions, HA is a biomaterial that shows promise in bone healing.\(^{[12]}\) On account of the fact that our study was conducted on rat mandibula to reflect the intramembranous ossification process and that it aimed to assess healing by applying HA on defects exclusively or in combination with bone graft, we can consider our study to be unique.

Various studies have been conducted on the effect of HA on the healing of bone defects formed in different regions of the body. Aslan et al.\(^{[13]}\) applied HA in combination with bone graft in the defect they formed in the rabbit tibia and they found fibrocartilage tissue and bone formation in the defect area after 40 days. In the study, Suzuki et al.\(^{[14]}\) came to the conclusion that the graft material that was used alongside HA specifically during the early stage of ossification improved the osteoconductive effectiveness. They also noted that HA played an active role in bone formation. In their study, Zou et al.\(^{[15]}\) noticed that bone formation in the group with only stem cell application was observed to be lower than in the group in which combined application of HA and stem cell. Bone healing findings obtained as a result of our study are consistent with the studies mentioned above. One of the major findings of our study is that the ossification levels in the group in which only HA was used and the one in which only bone graft was used are similar. The observation of fibrocartilage tissue formation in the group where HA is used alone supports the osteoinductive effect of HA. Although there are many studies in which HA is used with a graft or a biomaterial, the number of studies in which it is applied alone on bone defects like our study is limited. However, it was supported by other researchers that HA positively affected bone formation.\(^{[16,17]}\) For this reason, we think that although the amount of ossification seen in group 4 is not statistically significant compared with group 2 and group 3, it is still important in clinical terms. In addition to this, among the studies on bone healing, there are also some reporting that HA has no effect on bone healing. As a result of one of their studies, Segari et al.\(^{[18]}\) stated that HA had no superiority to bone grafts. He attributed the result of the study to the fact that HA used was not sufficient doses. One of the most important determinant of HA efficacy is its density. It is difficult to make a direct comparison between the density of the fibrous HA we used in our study and the density of HA used in the study in question. In our study, newly formed bone mineralization level was examined with microcomputed tomography. These results are one of the major findings in our study. HGA values were found in the areas at the defect border or near the defect borders. The groups in which HA was used (group 3 and group 4) showed no significant difference in terms of HGA values but had higher values than the other two groups. There was no significant difference between the remaining two groups (group 1 and group 2) in terms of HGA values. We think that high HGA values in areas near the defect margin is related to various factors including intense vascularization in said areas and defect boundary acting as a “roof” for mineralization. In their study, Pirc et al.\(^{[19]}\) stated that the high molecular weight HA plays a role in the formation of hydroxyapatite crystals by binding the Ca ion. In our study, HGA was found in the areas of the defect center or near the defect center. The highest LGA value belongs to the group where only HA is used. There was no statistical difference between the group 2 and 4, and they had a lower value than the group 3. The control group is the group with the lowest LGA value. The common theory regarding the mineralization of newly formed bone tissue is based on the release of the content of matrix vesicles contained within the osteoid.\(^{[20]}\) It can be considered that the ground substance formed in the region has an impact on the fact that level of mineralization observed in the near-center areas is the highest in the group 3. If the concentration of osteoid formed in near-center areas of the group 3 is higher than the other groups, then the level of mineralization in the region being higher than the other groups because of the effect of HA is an expected outcome. The presence of non-resorbed graft material supports this situation. The osteoconductive materials giving way to the bone tissue is only possible through the resorption of the bone graft. Since the graft materials are not completely resorbed, they occupy a physical place in the region. For this reason, the mineralization has occurred in resorbed cavities. Microcomputed tomography images show that in the group 2 bone grafts are found areas near the defect border and not in the areas near the center. In the group 4, the bone graft is homogeneously distributed. This situation supports the difference of HGA and LGA values between the groups. It can be considered that resorption took place in the near-center areas in the bone group 2 and that subsequently mineralization also started. However, the presence of bone grafts in areas near the defect borders indicates that no resorption occurred there. In the group 4, homogeneous distribution of graft material may be considered to provide equal resorption in all areas. The fact that the LGA value is lower in the groups where graft materials were used suggests that the bone graft was not fully resorbed. In the light of this information, it is possible to say that the HA turns into gel form after coming into contact with the blood and that it ensures the homogeneous distribution of the graft material because of its adhesive
property. The osteoconductive effect of the bone graft used is produced when it is replaced by new bone tissue during its resorption. However, this may take months depending on the size of the defect and the type of the graft material used. The HA we used in our study has a more stable structure than its equivalents produced in liquid form and takes longer to biodegrade. The HA molecules we used in our study are fully released and destroyed within 40 days. During this time, it acts as a place holder by turning into gel after coming into contact with blood. We think that the high molecular weight HA (with a molecular weight of 1300 kDa) we used plays a long-lasting and active role in bone production in the environment. This is expected to result in a decrease in osteoclastic activity in the region, especially with the presence of synthesized high molecular weight HA the site of new bone formation along the defect borders. We think that the long-term use of biomaterials with high molecular weight in the environment increases the osteoblastic activity and affects the osteoblast-derived osteoclastic maturation.

Alkaline phosphatase, osteopontin, and osterix markers, which play an active role in bone formation, turning out moderate/severe positive and the lack of any significant difference between the groups are among the other findings of our study.

The measurement method we use can be given as the reason for the fact that our findings related to the markers that reflect bone formation differ from the findings related to the histological bone healing. The density of the markers was measured subjectively using the Likert scale. We think that using numerical methods in measurement instead will yield more accurate results. Another issue that proved restrictive for our study is that the experiment period was determined as monochronic. If our study were conducted within two different experiment periods, we could have had the opportunity to assess the effectiveness of HA exclusively or combined with graft material during early and late recovery.

In the light of the findings obtained from our study, we can make a few recommendations for future experiments. We have indicated that the effectiveness of HA depends on its molecular weight and density. For this reason, new studies need to be conducted on the optimum density and molecular weight required for the repair of bone defects. We also recommend that the effectiveness of the HA used in the repair of bone defects on bone healing needs to be assessed at different time intervals, and its use needs to be assessed in combination with other biomaterials, not just bone grafts. There are very few clinical trials available regarding the HA used in our study. Although at the end of our studies, we concluded that HA plays a role in bone formation, we do not think the results are clinically sufficient. In this context, we expect that using HA in combination with other biomaterials or bone grafts in areas not exposed to direct forces such as sinus augmentation will yield more positive results.

The findings of our study suggest that the exclusive use of HA has at least as much an accelerating effect as the use of bone graft in the healing of the surgically created bone defects in the rat mandible, and that HA proves superior to bone grafts in the mineralization process of the defects. Whether used exclusively or in combination with bone grafts, HA is a promising biomaterial in repairing bone defects.

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There are no conflicts of interest.

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