Effects of Hyaluronic Acid and Hydroxyapatite/Beta-tricalcium Phosphate in Combination on Bone Regeneration of a Critical-size Defect in an Experimental Model

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Abstract: Hyaluronic acid (HyA) is an outstanding new product in the field of oral and maxillofacial surgery. The aim of this study was to evaluate the effects of HyA on bone regeneration in critical-size calvarial defects. Twenty-four female Sprague-Dawley rats were used in the present study. In each rat, 4 critical-size defects received different treatments: no treatment (control); HyA; Graft; and HyA + Graft combination. New bone formation, defect closure, inflammation, vascular proliferation, immature bone formation, mature bone formation, and bone marrow existence were investigated based on histological findings. The healing parameters related to bone formation (new bone formation, defect closure, immature bone formation) were significantly higher in the HyA group compared with the control group. However, HyA alone was unable to induce sufficient bone regeneration compared with treatments involving graft materials (Graft and HyA + Graft). In the Graft and HyA + Graft groups, prominent enhancement of all healing parameters was noted. The present results demonstrate that HyA alone did not adequately enhance bone regeneration in critical-size defects. Moreover, addition of HyA to a biphasic alloplastic graft material did not result in improved regeneration compared with the graft material alone.

Key Words: Biphasic bone graft, calvarial bone defect, hyaluronic acid

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Insufficient bone healing and quantity are consequences of tooth-related bone loss, such as traumatic tooth extraction, previous periodontal disease, or periapical pathology. Meanwhile, benign or malignant tumors that either originate in the jaws or spread to the jaws from other sites of the body can lead to a more severe bone loss. Bone defects that do not heal without interventions during the lifetime of the patient, either animal or human are defined as critical-size defects.[1]

Several biomaterials have been produced in recent years for the augmentation of bone defects, and bone graft materials are most frequently used for this purpose. Appropriate bone grafts should be osteoinductive, osteoconductive, osteogenic, without risk of infectious disease transmission, biocompatible, and bioresorbable. In addition, the bone graft material should have similar mechanical strength to the bone being replaced and should also be cost-effective.[2] Autogenous bone graft techniques remain the criterion standard for bone augmentation procedures because they provide an osteogenic, osteoinductive, and osteoconductive substrate for filling bone voids.[3] Although these techniques are often used for the treatment of bone defects that hamper the structural integrity of the jaws, they are restricted by the additional donor site morbidity and limited availability of graft material.[4] Allografts are an alternative to autogenous bone grafts for alveolar ridge augmentation with an advantage of avoiding donor site morbidity and related pain.[5] Xenografts, demineralized bone matrix, calcium phosphate, calcium sulfate, and bioactive glasses can also be used without donor site morbidity and are reported to enhance bone regeneration with their osteoconductive properties.[3] Furthermore, they are easy to obtain because of their unlimited availability.[3] Among osteoconductive graft materials, biphasic calcium phosphates came into prominence by allowing a better control over bioactivity and biodegradation that enhances the stability of the biomaterial while promoting bone ingrowth[6] and they can also be combined with bioactive molecules, therapeutic agents, and cells for other important applications such as tissue engineering, cell-therapy, and gene-therapy.[7] The osteoconductive properties of these graft materials allow cell attachment, proliferation, migration, and phenotypic expression of bone cells, leading to new bone formation on the surface of the biomaterials.[8]

Hyaluronic acid (HyA) is a naturally occurring biodegradable polymer of the extracellular matrix that is widely distributed throughout connective, epithelial, and neural tissues. HyA is a nonsulfated glycosaminogly can composed of repeating glucuronic acid and *N*-acetyl-D-glucosamine. Its negative charge and high hydration ratio play an important role in the control of tissue hydration.[9] HyA has both viscous and elastic properties owing to polymer chain entanglements.[10] The viscous properties provide lubrication, whereas the elastic properties have a shock-absorption effect.[11] HyA has found a number of usages in biomedical

applications as well as in cosmetics through its physiological functions and properties.[12]

HyA plays a pivotal role in wound healing. The creation of a hydrated and nonadhesive environment enhances cell migration, whereas, the interactions of proteoglycans with pericellular HyA impede migratory movements.[13] A HyA-rich environment decreases the proliferation rate of several types of stem cells. Meanwhile, the interactions of HyA with HyA-binding proteins mediate the maintenance and differentiation of these cells.[14] A HyA-rich environment provides a hydrated provisional wound matrix that is suitable for cell migration. Angiogenesis is a crucial necessity for appropriate wound healing, and the angiogenetic properties of HyA depend on its molecular weight. Previous experiments showed that high-molecular-weight HyA (HMW-HyA) in the extracellular matrix inhibits angiogenesis, whereas low-molecular-weight HyA (LMW-HyA) enhances angiogenesis and increases collagen production by endothelial cells.[15] In the maturation and remodeling phases of physiologic wound healing, HyA is produced in the wound margin and connective tissue matrix.[16]

Chang et al conducted an animals' study to investigate effects of hydroxyapatite/beta-tricalcium phosphate (HA-βTCP) alone and HyA/HA-βTCP combination on bone formation in the calvarial defects of rabbits and concluded that addition of HyA to HA-βTCP particles increase bone formation.[17] Generally, a commercial form of LMW-HyA (Tissue Support; Naturalize, Kassel, Germany) and a biphasic calcium phosphate ceramic combination (MBCP+; Biomatlante, Nantes, France) are particularly used for surgeries such as periimplant defects and sinus lift augmentation. The gel form of HyA can simplify the manipulation of particulate graft material, but there is little evidence in the literature to support its regenerative capacity and its consequences on bone healing during bone healing. Healing of graft material significantly influences the clinical success of treatment of bone defect, thus affecting the success and survival rate of implants if used for peri-implant bone augmentation. The aim of the present study was to evaluate the histopathological effects of HyA on inflammation and angiogenesis in addition to bone formation, both in the presence and absence of a graft material, in critical-size calvarial defects in a rat model.

MATERIALS AND METHODS

Animals and Surgical Procedure

The study was approved by the Baskent University Ethical Committee for Experimental Research on Animals (Project No: D-DA 13/09) and supported by the Baskent University Research Fund. Twenty-four female 12-week-old Sprague-Dawley rats were used as subjects in the study. The rats were kept on a 12-h/12-h day/night cycle and had *ad libitum* access to water and a standard laboratory diet.

Animals were anesthetized by intraperitoneal injection of a combination of ketamine (60 mg/kg) and xylazine (6 mg/kg). Preoperative enrofloxacin (10 mg/kg, intramuscular) and fentanyl (0.02 mg/kg, subcutaneous) were also given. The skin over the head was shaved and then disinfected with povidone-iodine. A full-thickness skin incision of approximately 2 cm was made along the sagittal suture of the skull. The subcutaneous tissues and periosteum were carefully dissected, and bone exposure was achieved. Four bilaterally symmetrical defects were created within the parietal and frontal bones (Fig. 1A). The variabilities caused by biological variation among animals were eliminated by the within-subject study design. The defect locations were determined by reference to the bregma point and sagittal suture in each animal. All defects were created with a standard 5-mm trephine bur and a low-speed handpiece under continuous irrigation with sterile saline. During the surgical procedure, attention was paid to keep the dura mater intact. Graft materials were used to fill specific defects in a planned manner. Specifically, 1 defect was left empty as the Control group, 1 defect was filled with only HyA gel (Tissue Support; Naturalize, Kassel, Germany), 1 defect was filled with only biphasic calcium phosphate ceramic (MBCP+; Biomatlante, Nantes, France), and the remaining 1 defect was filled with combination of HyA gel and biphasic calcium phosphate ceramic (Fig. 1B). The HyA used consisted of LMW-HyA gel containing sodium hyaluronate at 14 mg/mL. This commercial form of HyA was available in ready-to-use disposable sterile syringes, and the amount required to fill the bone defect completely was injected for the HyA group. The biphasic calcium phosphate ceramic consisted of 20% hydroxyapatite and 80% beta-tricalcium phosphate. The biphasic calcium phosphate ceramic was mixed with sterile saline and implanted to the bone defects created for Graft group. The HyA and graft materials were mixed in a sterile dappen dish and transferred to the bone defects created for the HyA + Graft group. The periosteum of the skull was sutured with absorbable 5.0 vicryl sutures to provide complete periosteal coverage of the bone defects and materials. Finally, the skin was closed with 4.0 silk sutures. A postoperative antibiotic (enrofloxacin, 10 mg/kg, intramuscular, twice daily) and analgesic (fentanyl, 0.02 mg/kg, subcutaneous, twice daily) were administered for 5 days to prevent infection and provide analgesia.

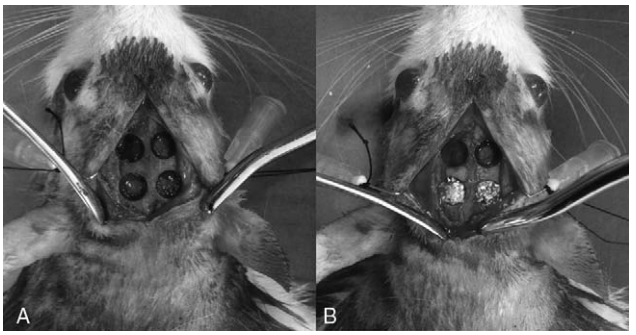

FIGURE 1. Surgical photos during bone defect creation and augmentation with materials. (A) Four bilaterally symmetrical defects were created within the parietal and frontal bones. The defect locations were determined by reference to the bregma point and sagittal suture in each animal. (B) The anterior defect on the right side was left empty as a Control sample and the anterior defect on the left side was filled with only hyaluronic acid (HyA) gel. The posterior defect on the right side was filled with only graft material and the left posterior defect was filled with the combination of HyA and graft material.

At the end of postoperative week 4, the rats were randomly divided into 2 groups, and the rats in the first group (n = 12) were euthanized with an overdose of thiopental sodium (150 mg/kg). The rats in the second group (n = 12) were euthanized at the end of postoperative week 8 in the same manner as followed in the first group. Thereafter, the skulls of the animals including all bone defects were removed.

Histopathological Analysis

The skulls of the rats were fixed with 10% phosphate-buffered formaldehyde for 2 days, decalcified with 5% formic acid solution for 2 weeks, dehydrated with a graded series of ethyl alcohol and xylene, and were finally embedded in paraffin blocks. The calvaria were sectioned perpendicular to the sagittal suture, passing through the center of the defects. Four central sections stained with hematoxylin–eosin were used to assess new bone formation, defect

TABLE 1. Four Histological Sections of Each Sample Was Semiquantitatively Evaluated By a Pathologist, Blinded to the Type of Treatment. Each Parameter Was Rated According to the Scoring System in the Table During Histological Investigation of the Samples

1- New bone formation	2- Defect closure
0: No bone formation	0: No defect closure
1: <25% bone formation	1: <25% closure
2: 25–50% bone formation	2: 25–50% closure
3: 50–75% bone formation	3: 50–75% closure
4: 75–100% bone formation	4: 75–100% closure
3- Inflammation	4- Vascular proliferation (400× magnification)
0: No inflammation	0: No vessels
1: Mild inflammation	1: <10 vessels
2: Moderate inflammation	2: 10–20 vessels
3: Severe inflammation	3: >20 vessels
5- Immature component of bone tissue	6- Mature component of bone tissue
0: Absent	0: Absent
1: <25%	1: <25%
2: 25–50%	2: 25%–50%
3: 50–75%	3: 50%–75%
4: 75–100%	4: 75%–100%
7- Bone marrow existence	
0: Absent	
1: Present	

closure, inflammation, vascular proliferation, bone marrow existence, and immature and mature components of new bone. All histopathological analyses were performed under a light microscope (Model BX53; Olympus, Hamburg, Germany) by a pathologist blinded to the type of treatment, and the parameters were evaluated semiquantitatively. The study samples were evaluated according to the measurements listed in Table 1. To determine vascular proliferation, small vascular structures were counted in 3 high-magnification fields (×400) in each defect. Inflammation was evaluated in all fields and scored as follows: mild, inflammatory cell infiltration in <25% of the defect area; moderate, inflammatory cell infiltration in 25% to 50% of the defect area; and severe, inflammatory cell infiltration in >50% of the defect area. Newly formed osteoid matrix with osteoblastic rimming was defined as the immature component of new bone formation and scored in all fields of the defects. Lamellar bone structures were defined as the mature component of new bone tissue and scored in a similar manner to the immature component.

Statistical Analysis

Sample size was calculated by a power analysis to ensure at least 80% power with 0.05 significance level for the study. According to the results of the power analysis, 12 rats in each group would provide 82% power for the study. Statistical analyses were carried out using PASW 18.0 (SPSS Inc, Chicago, IL). The significance of differences between samples was examined by the nonparametric Kruskal–Wallis test. The Mann–Whitney *U* test was used for post-hoc comparisons between the 2 treatment samples. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

All rats survived until the end of the study, and no postoperative complications were noted such as bone or graft exposure, infection, and allergic reaction. The mean ± SD values for all parameters in the samples and the corresponding *P* values are presented in Table 2.

TABLE 2. Mean ± SD Values for all Parameters in the Samples and *P* Values Obtained by the Kruskal–Wallis test. All of the Provided Data Are Based on the Scales Shown in Table I. Graft Augmented Samples (Graft, HyA + Graft) Showed Better Results For Each Evaluated Parameters, Except Bone Marrow Existence After 4 Weeks of Healing Period. In Addition to Bone Marrow Existence, Inflammation Results Were Similar Between Samples at the End of 8 Weeks of Healing and Other Parameters Were Significantly Higher in the Graft Augmented Samples (Graft, HyA + Graft) as After 4 Weeks of Healing

	4 Weeks Postoperatively					8 Weeks Postoperatively				
Histopathologic Parameters	**Control Samples**	**HyA Samples**	**Graft Samples**	**HyA + Graft Samples**	***P***	**Control Samples**	**HyA Samples**	**Graft Samples**	**HyA + Graft Samples**	***P***
New bone formation	0 ± 0	0.7 ± 0.43	2.58 ± 1.31	3.41 ± 0.9	<0.001	0.2 ± 0.42	0.8 ± 0.63	2.66 ± 0.49	2.58 ± 0.66	<0.001
Defect closure	0 ± 0	0.7 ± 0.48	3.33 ± 1.15	3.83 ± 0.38	<0.001	0.3 ± 0.48	0.9 ± 0.56	3.66 ± 0.77	3.75 ± 0.62	<0.001
Inflammation	1 ± 0	1 ± 0	1.66 ± 0.49	1.75 ± 0.45	<0.001	1.6 ± 0.51	1.6 ± 0.51	1.41 ± 0.51	1.5 ± 0.52	0.796
Vascular proliferation	0.9 ± 0.3	1.2 ± 0.42	2.08 ± 0.51	2.25 ± 0.45	<0.001	1.6 ± 0.51	1.3 ± 0.48	2.08 ± 0.66	2.5 ± 0.67	0.001
Immature component of bone tissue	0 ± 0	2 ± 2.1	3.33 ± 1.15	3.16 ± 0.93	<0.001	0.8 ± 1.68	2.8 ± 1.93	2.75 ± 1.13	3 ± 1.12	0.017
Mature component of bone tissue	0 ± 0	0 ± 0	1.25 ± 0.86	0.83 ± 0.71	<0.001	0 ± 0	0 ± 0	1.25 ± 0.96	1.58 ± 1.24	<0.001
Bone marrow existence	0 ± 0	0 ± 0	0.16 ± 0.38	0.25 ± 0.45	0.156	0 ± 0	0 ± 0	0.5 ± 0.52	0.16 ± 0.38	0.006

HyA, hyaluronic acid.

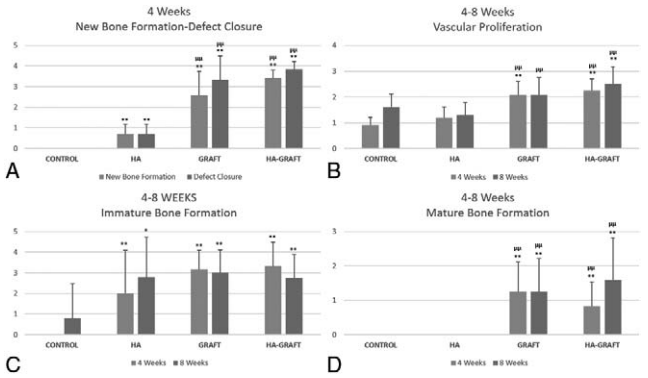

FIGURE 2. Histopathological result graphs for some of the parameters. (A) New bone formation and defect closure scores of all samples after 4 weeks of healing. (B) Vascular proliferation scores of all samples after 4 and 8 weeks of healing. (C) Immature bone formation scores after 4 and 8 weeks of healing. (D) Histopathological mature bone formation scores of different treatment samples after 4 and 8 weeks of healing. $*P<0.05$, $**P<0.01$, versus Control samples. $^{\#}P<0.05$, $^{\#\#}P<0.01$, versus hyaluronic acid samples.

New Bone Formation

The histopathologic analysis of the 4-week samples showed higher new bone formation within the HyA ($P=0.001$), Graft ($P<0.001$), and HyA + Graft ($P<0.001$) calvarial defects compared with the Control defects. Without any treatment, no bone formation was observed in the critical-size calvarial defects after 4 weeks. The new bone formation results for the Graft ($P<0.001$) and HyA + Graft ($P<0.001$) samples were significantly higher than those for the HyA samples. However, no significant differences were detected between the Graft and HyA + Graft samples ($P=0.091$) (Fig. 2A). The distribution of new bone formation across groups at 8 weeks was similar to those at 4 weeks. Bone formation in the calvarial defects was significantly greater in the HyA ($P=0.025$), Graft ($P<0.001$), and HyA + Graft ($P<0.001$) samples compared with the Control samples at 8 weeks. In addition, the Graft ($P<0.001$) and HyA + Graft ($P<0.001$) samples had significantly more new bone formation compared with the HyA samples. However, the Graft and HyA + Graft samples had similar results, and the differences were not significant at 8 weeks ($P=0.599$).

Bone apposition was noticeable on the surface of the graft particles (Fig. 3A), and new bone formation was centripetal from the host bone in the margin of the defects. Bone formation in the non-grafted samples was in the form of bone islands and had immature characteristics with the presence of surrounding cuboidal osteoblasts (Fig. 3B).

Defect Closure

The defect closure scores relevant to new bone formation and the similarities between the defect closure and new bone formation scores after 4 weeks of healing are presented in Fig. 2A. After 4 weeks of healing, the histopathologic analysis showed significantly higher defect closure in the HyA ($P=0.001$), Graft ($P<0.001$), and HyA + Graft ($P<0.001$) samples compared with the Control samples. In addition, defect closure was significantly higher in the Graft ($P<0.001$) and HyA + Graft ($P<0.001$) samples than in the HyA samples. There were no significant differences between the Graft and HyA + Graft samples ($P=0.167$). The results at 8 weeks showed significantly higher defect closure in the HyA ($P=0.024$), Graft ($P<0.001$), and HyA + Graft ($P<0.001$) samples compared with the Control samples. The defect

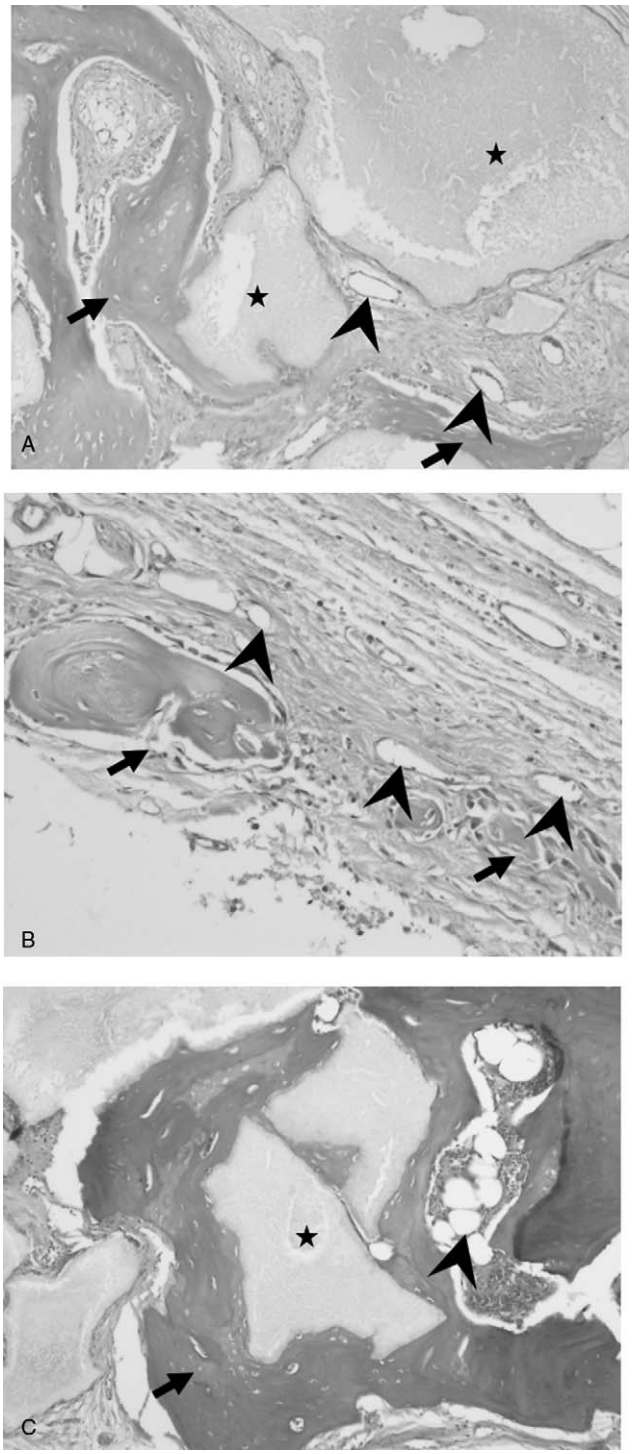

FIGURE 3. Representative hematoxylin–eosin stained histopathological sections under ×200 magnification. (A) Blood vessels (black arrowheads), graft particles (asterisks), and bone formation around graft particles (black arrows) are shown in a histopathological section from the hyaluronic acid (HyA) + Graft group after 4 weeks of healing. (B) One of the tissue sections in the HyA group after 8 weeks of healing, showing bone islands in the bone defect (black arrows) and blood vessels (black arrowheads). Osteoblasts can be seen around the immature bone islands. The little bone formation in the HyA group can be attributed to the absence of the osteoconductive effects of graft particles. (C) Mature bone formation (black arrow) around a graft particle (asterisk) and bone marrow existence (black arrowhead) are noted in a histopathological section from the Graft group after 8 weeks of healing.

closure scores for the Graft ($P < 0.001$) and HyA + Graft ($P < 0.001$) samples were significantly higher than those for the HyA samples. However, the Graft and HyA + Graft groups had similar results, and the differences were not significant ($P = 0.929$).

Inflammation

The histopathologic analysis revealed higher inflammation in the Graft ($P = 0.001$) and HyA + Graft ($P < 0.001$) samples compared with the Control samples after 4 weeks of healing. In addition, the results of inflammation for the Graft ($P = 0.002$) and HyA + Graft ($P = 0.001$) samples were significantly higher than those for the HyA samples. However, there were no significant differences between the HyA and Control samples ($P = 1$) and between the Graft and HyA + Graft samples ($P = 0.66$). After 8 weeks of healing, the mean inflammation scores were very similar, and there were no significant differences between any types of treatments ($P = 0.796$).

Vascular Proliferation

The histopathological analysis of vascular proliferation showed significantly increased numbers of vessels in the Graft and HyA + Graft samples compared with the HyA and Control samples after 4 weeks of healing ($P = 0.001$ for all comparisons). However, no significant differences were detected between the Control and HyA samples ($P = 0.083$) as well as between the Graft and HyA + Graft samples ($P = 0.422$). Comparisons of the 8-week samples revealed a significantly higher vessel density in the HyA + Graft samples compared with the HyA ($P = 0.001$) and Control ($P = 0.005$) samples. In addition, a significantly higher vessel number was observed in the Graft samples compared with the HyA samples ($P = 0.009$). Meanwhile, there were no significant differences between the Control and HyA samples ($P = 0.189$), Control and Graft samples ($P = 0.084$), and Graft and HyA + Graft samples ($P = 0.121$) (Fig. 2B). Vessels in tissue sections from the different samples are presented in Fig. 3A and B.

Immature Bone Formation

The histopathological analysis revealed higher immature bone formation in the HyA ($P = 0.009$), Graft ($P < 0.001$), and HyA + Graft ($P < 0.001$) samples compared with the Control samples at 4 weeks. The analysis revealed higher scores in the Graft and HyA + Graft samples compared with the HyA samples, but the differences were not significant. After 8 weeks of healing, the immature bone formation was significantly greater in the HyA ($P = 0.028$), Graft ($P = 0.006$), and HyA + Graft ($P = 0.004$) samples compared with the Control samples, similar to the findings at 4 weeks. The differences between the HyA, Graft, and HyA + Graft samples were not significant (Fig. 2C). The immature bone formation in the HyA samples was typical, with the formation of bone islands (Fig. 3B) and centripetal bone growth from the surrounding host bone.

Mature Bone Formation

No mature bone formation was observed in the Control and HyA samples after both 4 and 8 weeks of healing. Treatments that included graft materials were capable of inducing mature bone formation after 4 and 8 weeks of healing. Histopathologic investigation of the 4-week samples indicated that the Graft ($P = 0.001$) and HyA + Graft ($P < 0.001$) samples had significantly greater mature bone formation than the Control and HyA samples, but the differences between the Graft and HyA + Graft samples were not significant ($P = 0.235$). After 8 weeks of healing, the Graft and HyA + Graft samples had significantly greater mature bone formation than the Control and HyA samples ($P = 0.001$). In contrast, the Graft and HyA + Graft samples had similar mature bone formation scores, and no significant difference was observed ($P = 0.507$) (Fig. 2D). The mature bone formation was observed only in the defects in the Graft and HyA + Graft groups. The mature bone was typically observed around the graft materials, and the bone formation in the other samples was in the immature form (Fig. 3C).

Bone Marrow Existence

Bone marrow existence was only observed in the graft-augmented defects (Graft and HyA + Graft groups) at both 4 and 8 weeks of healing. At 4 weeks, no significant differences were detected in the samples ($P = 0.156$). However, at 8 weeks, significantly higher bone marrow existence was observed in the Graft and HyA + Graft samples compared with the Control ($P = 0.01$) and HyA ($P = 0.01$) samples. Bone marrow was observed within the remodeled mature bone in the Graft and HyA + Graft samples (Fig. 3C).

DISCUSSION

The results of the present study indicated that HyA enhanced bone formation in the critical-size calvarial defects when used alone. Meanwhile, bone formation was more noticeable in the Graft and HyA + Graft defects compared with the HyA defects. The in vitro and in vivo experiments of Nguyen and Lee[18] revealed enhanced bone regeneration and cell proliferation around a microspongeHyA-gel material. However, in the present study, HyA was used with a biphasic calcium phosphate scaffold. Aslan et al[19] stated that bone formation in noncritical-size tibial defects filled with HyA plus an allogenic cancellous bone graft was higher than that in defects filled with the allogenic cancellous bone graft alone at the late stage of healing. However, there may be important differences in the healing process in non-critical-size bone defects compared with critical-size defects such as those analyzed in the present study. To clarify these differences, more research should be performed on noncritical-size bone defects. Similar results as reported by Aslan et al were also observed by Schwartz et al[20] in a clinical study showing that HyA was capable of inducing bone formation as a sinus lift material, but had less effect than its combination with bone graft materials. Study of Chang et al[17] investigated newly formed bone by micro CT and showed that HyA, HA-βTCP, and combination of both enhanced bone formation in the rabbit calvarial defects. However, HA-βTCP-filled defects had similar results with defects filled with a combination of HyA and HA-βTCP. Results of the previous study strongly corroborate our findings on new bone formation. According to the aforementioned previous studies and our present results, a scaffold or graft material that provides osteoconductive properties is essential during HyA usage in the field of bone regeneration. The mature bone formation in the Graft and HyA + Graft samples was also remarkable. There was no mature bone formation in the Control and HyA samples, even after 8 weeks of healing. The study by Aguado et al[21] showed that HyA was unable to restore the trabecular microarchitecture of critical-size femoral defects and that the new bone volume to total defect volume ratio was also low.

An inflammatory response is a hallmark of the initial response of the body to injury. Inflammatory cells can accelerate the repair process by providing microbial decontamination, whereas anti-inflammatory cytokines and multiple growth factors synthesized by macrophages at the late stage of inflammation are critical for wound healing.[22] In a previous study investigating the effects of HyA on mesenchymal stromal cell culture, it was reported that HMW-HyA increased the ratios of secreted interleukin-10/interferon-gamma and interleukin-10/interleukin-2 that are characteristics of the

anti-inflammatory stage of wound healing.[23] However, endogenous LMW-HyA in the extracellular matrix acts like lipopolysaccharide, which is produced during infection and functions as a proinflammatory mediator.[24] Ruppert et al[25] stated that HMW-HyA decreases the production of inflammatory cytokines and impairs the phagocytosis of lymphocytes. The interaction of HMW-HyA and CD44 appears crucial for these anti-inflammatory properties. Whereas, in another study, intrapleural injection of HyA-modulated leukocyte migration and resolved neutrophilic inflammation.[26] However, the study observed that the same materials had no modulatory effect on the inflammatory process during bone repair of tooth sockets. In our results, the inflammatory cell infiltration into the HyA defects was the same as that into the Control defects after 4 weeks of calvarial bone defect healing. More infiltration was observed in the Graft and HyA + Graft defects, but the difference between these two samples was not significant. After 8 weeks of healing, all samples had similar results with regard to inflammation (Table 2). Although the anti-inflammatory effects of HMW-HyA and inflammatory effects of LMW-HyA were demonstrated in different studies, it is clear that the effects of HyA depend on the applied tissue, molecular weight, and production specifications.[27] To the best of our knowledge, there is no report available regarding the anti-inflammatory properties of HyA during bone healing. However, in line with the present study, histopathologic analysis revealed no effect of HyA on the inflammatory process during tooth socket healing.[26] The cell count in a tissue section is the primary parameter for histologic and histomorphometric analyses. However, the anti-inflammatory effects of HyA are mediated by molecular interactions. Thus, the underlying mechanisms that regulate the inflammatory or anti-inflammatory effects of HyA during bone healing have not been fully defined, and remain an unexplored area for further examination.

Nutrients, oxygen, growth factors, cytokines, osteoblasts, and osteoclasts are supplied to the metabolically active regenerating callus by the formation of new blood vessels.[28] Therefore, blood flow to a bone defect is crucial for appropriate bone healing. The term "angiogenic–osteogenic coupling" refers to the close spatial and temporal association of bone formation with vascularization.[29] Early vascularization is the first step of the association and is essential for osteogenic repair of critical-size bone defects by providing nutritional support for bone grafts.[30] Angiogenesis occurs primarily before the beginning of osteogenesis, and the newly formed blood vessels ensure steady transport of osteoblast and osteoclast precursors to the remodeling bone.[31] Hyaluronidases in wounds degrade HMW-HyA to LMW-HyA, and Takahashi et al[32] showed that HyA fragments (6 disaccharides in length) increased endothelial sprouting. Slevin et al[33] described that oligosaccharides of HyA induced endothelial cell proliferation and wound recovery. Moreover, Galeano et al[34] used genetically diabetic mice during an investigation of the systemic effects of HMW-HyA on skin healing. Their histological and immunohistochemical investigations indicated that systemic HMW-HyA enhanced angiogenesis within the skin incisions. Meanwhile, bone grafts serve as scaffolds for growing or newly formed blood vessels. The success of a bone graft depends on its ability to induce and support vascular infiltration. The size, shape, and porosity of alloplastic bone grafts are determinants of their biomaterial integration with host tissues and angiogenesis.[35] The present histologic investigation indicated that after 4 and 8 weeks of healing, no significant differences were observed between the Control and HyA samples or between the Graft and HyA + Graft samples with regard to vascular proliferation. However, vascular proliferation was higher in the bone defects treated with the graft materials at both observation points (Table 2). According to our results, LMW-HyA seems to be ineffective for angiogenesis during treatment of critical-size calvarial bone defects. In critical-size defects, a scaffold for endothelial ingrowth is necessary and HyA could not achieve this ingrowth when used alone. However, combining LMW-HyA with a graft material did not adversely influence the angiogenesis in the bone defects. The similarity between the vascular proliferation scores of the groups at 4 and 8 weeks revealed that the majority of the angiogenesis occurred within the first 4 weeks of bone healing.

HyA influences the cellular mechanisms during regenerative processes, and in the present study, histopathological examination was used to determine the effects of HyA. However, more detailed studies on the effects of HyA during the regeneration of critical-size bone defects should be explored with more sophisticated investigation methods.

Within the limitations of the present study, it can be concluded that commercially available LMW-HyA has limited effects on bone formation in critical-size bone defects and has no effect on the inflammation and angiogenesis of the healing tissue. Graft augmentation for the treatment of bone defects enhances both angiogenesis and bone formation, and leads to the acquisition of bone maturity and appropriate microarchitecture essential for the physiological function of bone. The present findings corroborate the idea that critical-size bone defects require a graft for proper bone healing. Furthermore, the present study indicates that HyA gel can simplify the manipulation of graft particles during clinical use without reducing the regenerative properties of alloplastic graft materials.

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