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ARAŞTIRMA / RESEARCH

Effects of hyaluronic acid and gamma-radiated mineralized allografts on the healing of rat tibial defects

Hyaluronik asit ve gama radyasyonlu mineralize allogreftlerin sıçan tibial defektlerinin iyileşmesi üzerine etkileri

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Abstract

Purpose: This study aimed to evaluate the effects of hyaluronic acid (HyA) and gamma-radiated mineralized allografts (Gr-MAs) on the healing of bone defects in rat tibiae.

Materials and Methods: Fifty-two male Sprague Dawley rats were randomly allocated to four groups: Gr-MA, HyA, Gr-MA combined with HyA (Gr-MA + HyA), and controls with empty defects. The animals were sacrificed on the 7th and 21st postoperative days. The inflammation, necrosis, fibrosis, new bone formation, and bone healing scores were evaluated on the basis of the histopathological findings.

Results: The amount of new bone formation was found to be significantly greater in the control group than in the experimental groups. In addition, the healing scores were statistically higher in the control and the Gr-MA + HyA groups. Comparisons of the control, graft, and HyA groups indicated that the control group exhibited significantly less necrosis on the 7th day; however, on the 21st day, there were no statistically significant differences among the groups. There were no statistically significant differences among the groups in terms of the inflammation and fibrosis levels on the 7th or 21st days.

Conclusion: Within the limitations of this study, the application of HyA alone and the addition of HyA to Gr-MA did not improve bone regeneration in rat tibial defects.

Keywords: Hyaluronic acid, bone grafting, bone regeneration, bone formation, rats

Öz

Amaç: Hyaluronik asidin ve mineralize allogreftin sıçan tibiasında oluşturulmuş defektlerde yeni kemik formasyonu ve kemik iyileşme skoru üzerine etkisinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: 52 adet Spraque-Dawley cinsi sıçan 4 gruba ayrılmıştır: mineralize kemik greft grubu, hyaluronik asit grubu, hyaluronik asit ile kombine olarak uygulanan mineralize kemik greft grubu ve boş defektlere sahip kontrol grubu. Hayvanlar postoperatif 7. ve 21. günlerde sakrifiye edilmiştir. İnflamasyon, nekroz, fibrosis, yeni kemik oluşumu ve kemik iyileşme skoru histopatolojik olarak değerlendirilmiştir.

Bulgular: Yeni kemik oluşumu kontrol grubunda deney grubuna göre anlamlı oranda daha yüksek bulunmuştur. Ayrıca yalnızca hyaluronik asit ve yalnızca greft gruplarına kıyasla, kontrol grubunda iyileşme skoru daha yüksek bulunmuştur. Greft ve hyaluronik asit grupları kıyaslandığında, 7. gündeki nekroz kontrol grubunda anlamlı oranda düşükken, 21. günde gruplar arasında anlamlı bir farklılık bulunmamıştır. 7. ve 21. günlerdeki inflamasyon ve fibrozis değişkenlerinin oranları gruplar arasında anlamlı bir değişiklik yaratmamıştır.

Sonuç: Hyaluronik asit tek başına veya mineralize kemik allogrefti ile birlikte uygulandığında, sıçan tibiasında oluşturulmuş kritik boyutta olmayan defektlerde kemik rejenerasyonununda yeterli katkıyı sağlamamıştır.

Anahtar kelimeler: Hyaluronik asid, kemik greftleme, kemik yenilenmesi, kemik oluşumu, ratlar

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INTRODUCTION

Bone graft materials are commonly used as a void filler for bone defects[1-5]. Several treatment methods, such as the application of local and systemic drugs, graft materials, hormones, growth factors, bone morphogenic proteins, physical stimulation, and hyperbaric oxygen, have been used to accelerate the healing of bone defects and fractures[2-4]. The choice of bone graft material plays a crucial role in bone grafting augmentation techniques. Bone grafts can be classified as autogenous bone, allografts, xenografts, and synthetic[5]. Autogenous bone grafts are still currently accepted as the gold standard for bone augmentation because of their osteogenicity, osteoinductivity, and osteoconductivity. However, because of the limitations of autogenous grafts, several bone substitutes have been introduced.

Allografts are tissues that are taken from donors of the same species as the host. Recently, gamma-radiated mineralized allografts (Gr-MAs) have become another preferred treatment that promotes rapid healing and offers complete remodeling[5]. Gr-MAs have exhibited better adaptation to the surrounding tissues because of the variations in tissue banks' allograft processing methods[6]. Mineralized allografts and their mineral content provide better volume stability at the grafting site than do other allografts[7].

Hyaluronic acid (HyA) is one of the important components of the extracellular matrix of tissues. It is a naturally derived linear high-molecular-weight protein with viscoelastic properties[8]. HyA contributes to bone formation and prior osteogenic commitments by regulating cytokines and growth factors[9-12]. These are some of the basic characteristics of HyA that can mediate both acute and chronic wound healing. Several studies have demonstrated the effects of bone grafts and HyA on bone healing; however, few have provided comparisons of these materials.

The aim of the present study was to evaluate the histopathological effects of HyA and Gr-MA on the new bone formation and bone healing processes in tibial defects in rats. The null hypothesis tested in the present study is that hyaluronic acid (HyA) and Gr-MA would not contribute to new bone formation and healing when applied together or alone to rat tibial defects.

MATERIALS AND METHODS

Fifty-two male 10–12-week-old Sprague Dawley rats weighing 350–400 g were used in the study. All of the animals were randomly allocated to four groups, each with a different augmentation material for treating the tibial defects. Three experimental groups, each consisting of 16 animals, received the following treatments: The first group received only HyA (HYALOSS™ Matrix; Anika Therapeutics, Padova, Italy), which was applied to the bone defect. The second group received Gr-MA (Puros® allograft; Centerpulse Dental Division, Carlsbad, CA, USA), which was applied to the bone defect. The third group received Gr-MA combined with HyA (Gr-MA + HyA). In the control group, which consisted of four animals, the bone defect was irrigated with a sterile saline solution.

Anesthesia and surgical procedure

This study was approved by the Institutional Animal Care and Ethics Committee of Istanbul University Institute for Experimental Medical Research (Project No 2014/92, Date of Ethical Approval 09/10/2014). Experimental protocol has been completed in accordance with the Guide for the Care and Use of Laboratory Animals. The animals were anesthetized by the intraperitoneal injection of a combination of 60 mg/kg ketamine (Ketalar; Eczacibaşi-Warner Lambert, Istanbul, Turkey) and 6 mg/kg xylazine (Rompun® 2%; Bayer, Istanbul, Turkey). Preoperatively, the skin over the tibia was shaved and disinfected with povidone-iodine. The bone was exposed through a full-thickness skin incision of approximately 2 cm. A right tibial non-critical bone defect with a diameter of 3 mm was prepared with a dental handpiece and a trephine bur under copious saline irrigation (Figure 1). The wounds were closed with 3-0 silk sutures. Half of the animals in each group were sacrificed on the 7th postoperative day, and the other half were sacrificed on the 21st postoperative day.

Histopathological evaluation

The tibiae were removed and fixed in 10% phosphate-buffered formaldehyde for 1 week. After fixation, the material was decalcified in a formic acid sodium nitrate solution. Paraffin tissue blocks were then prepared and deparaffinized. After staining with hematoxylin and eosin, the sections were examined under a light microscope (Olympus BX60; Olympus

Optical Co. Ltd., Tokyo, Japan) attached to a color video camera that was connected to a personal computer. The images were captured, and the parameters of interest were measured with analySIS FIVE software (Olympus Optical Co. Ltd.) at 100× magnification. Digital images were obtained from the tissue sections of all the defect areas. These areas were analyzed for inflammation, necrosis, fibrosis, new bone formation, and bone healing. The inflammation and fibrosis were assessed as follows: 0 (–), 1 (1–30%), 2 (30–60%), and 3 (>60%)[13]. Micro-abscess formation was also scored as 3. Bone healing was evaluated on the basis of Allen's[14] fracture healing scores, including the healing stages: non-union—0, incomplete cartilage union—1, complete cartilage union—2, incomplete bony union with phase of ossification—3, incomplete bony union with intermediate phase of ossification—4, incomplete bony union with late phase of ossification—5, and complete bony union—6. The areas occupied by newly formed bone and fibrosis were measured, and the proportion (%) with respect to the total area was determined. Necrosis and inflammation were scored as present (−) or absent (+).

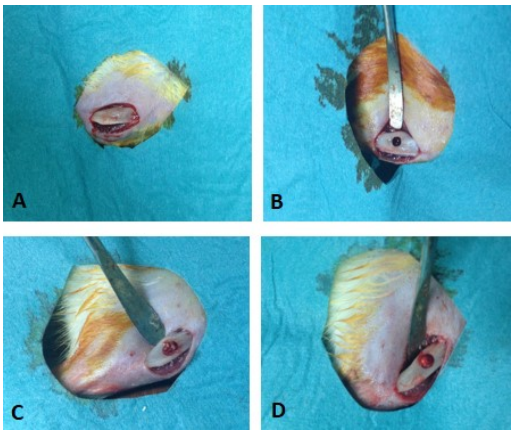

Figure 1. Surgical procedures. (A) Rat tibia after dissection; (B) Defect; (C) Application of gamma-radiated mineralized bone graft; (D) Application of hyaluronic acid.

Statistical analysis

IBM SPSS Statistics for Windows, Version 22 software (IBM Corp., Armonk, NY, USA) was used. Descriptive statistics, such as the median, standard deviation, and frequency, were used. The group variables that were normally distributed were evaluated with the Shapiro–Wilk test, and those that were not normally distributed were evaluated with the Kruskal–Wallis test. Levene's test was used for the homogeneity of variances. The Mann–Whitney U test was performed for pairwise comparisons. The Fisher–Freeman–Halton test and Fisher's exact test were used for the analysis of the categorical variables. The confidence interval was set to 95%, and $p < 0.05$ was considered statistically significant.

RESULTS

Day 7 assessment

In the HyA group, active fibrous connective tissue was observed in the defect area, and in the Gr-MA group, new bone formation was seen around the graft material. In the Gr-MA + HyA group, new trabecular bone was observed to be filling the defect region around the active fibrous connective tissue. In the control group, active fibrous connective tissue was observed around the new bone formation in the defect area (Figure 2).

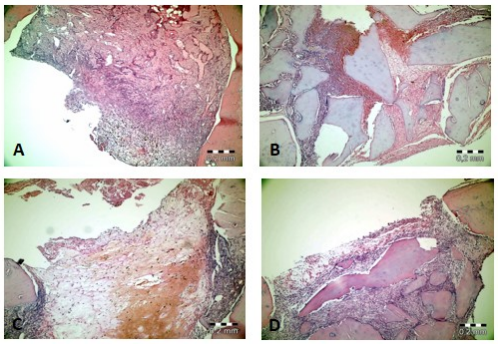

Figure 2. Representative hematoxylin-eosin-stained histopathological sections under 100× magnification on the 7th day. (A) In the control group, new bone formation in and around the active fibrous connective tissue in the defect region; (B) In the gamma-radiated mineralized allograft (Gr-MA) group, new bone trabeculae around the graft material covering large areas of the active connective tissue; (C) In the hyaluronic acid (HyA) group, active fibrous connective tissue around the defect; (D) In the Gr-MA combined with HyA group, the presence of large graft particles in the fibrous connective tissue in the defect region and bone formation around the graft particles.

The necrosis levels were found to be significantly lower in the control group than in the HyA and the Gr-MA groups ($p = 0.022$). The inflammation levels

in the Gr-MA and the control groups were statistically higher on the 7th day than on the 21st day (p = 0.003).

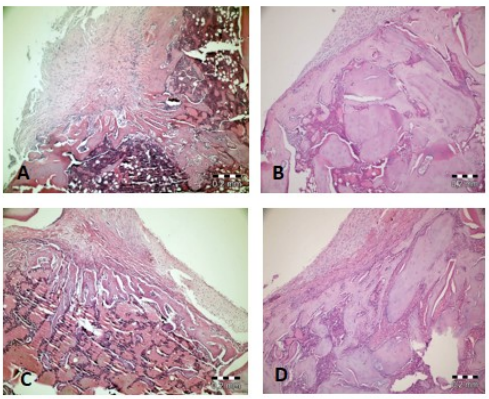

Figure 3. Representative hematoxylin-eosin-stained histopathological sections under 100× magnification on the 21st day. (A) In the control group, new bone formation filling the defect site and active fibrous connective tissue around the defect; (B) In the gamma-radiated mineralized allograft (Gr-MA) group, new bone formation around the graft material; (C) In the hyaluronic acid (HyA) group, new bone formation in the fibrous connective tissue; (D) In the Gr-MA combined with HyA group, new bone formation covering the defect surface.

Similar to the Gr-MA and the control groups, the Gr-MA + HyA group exhibited significantly higher inflammation levels on the 7th day than on the 21st day inflammation (p = 0.001). The control group had a significantly greater amount of new bone formation than the HyA group (p = 0.025), the Gr-MA group (p = 0.015), and the Gr-MA + HyA group (p = 0.039; Table 1).

The healing scores for the control group were significantly higher than those for the HyA group (p = 0.007), graft group (p = 0.002), and Gr-MA + HyA group (p = 0.004). There were statistically significant differences in the necrosis levels of the groups on the 7th day (p = 0.005).

Day 21 assessment

New bone formation around the graft material was observed in the Gr-MA group, and in the HyA group, bone islands were formed in the fibrous connective tissue. In the Gr-MA + HyA group, new bone formation filled the defect area, and there was residual graft material in the deep tissues. In the control group, fibrous connective tissue around the new bone formation filled the defect area (Figure 3).

The control group exhibited a significantly greater amount of new bone formation than the Gr-MA group (p = 0.028) and the Gr-MA + HyA group (p = 0.048). In all the groups, the amount of new bone formation was significantly greater on the 21st day than on the 7th day. On the 21st day, there were statistically significant differences in the healing scores (p = 0.001). The control group's healing scores were significantly higher than those of the HyA and the Gr-MA groups (p = 0.006). For all the groups, the healing scores on the 21st day were significantly higher than those on the 7th day (Table 1).

Table 1. Healing scores and new bone formation on the 7th and 21st postoperative days

		Control	Graft	Hyaluronic Acid	Graft + Hyaluronic Acid	p
		Median	Median	Median	Median	
New bone formation	7th day	0.11 A	0.04 a	0 a	0.08 a	0.027*
	21st day	0.43 A	0.14 a	0.27 a	0.29 a	0.049*
	P	0.009**	0.046*	0.001**	0.001**	
Healing score	7th day	3 A	1 a	0 a	1 a	0.003**
	21st day	5 A	2.5 a	4 a	5 a	0.001**
	P	0.005**	0.001**	0.002**	0.001**	

DISCUSSION

This study was conducted to evaluate the effects of HyA and Gr-MA on the healing of bone defects. Allografts, which provide type I collagen and contain bone morphogenic proteins, are commonly used as void fillers in maxillofacial bone defects[15]. The Puros® allograft, a two-piece graft composed of cortical and cancellous bone, is obtained mostly from the ends of the long bones, such as the humeral head, femoral head, femoral condyles, and tibial plateau.

This graft material receives low-dose gamma irradiation, which ensures biomechanical integrity, preserves protein structure, and inactivates all remaining viruses[14]. HYAFF®-11, an esterified form of HyA, forms a scaffold for cell growth[16]. The HYALOSS Matrix is composed of bundles of fibers made entirely of HYAFF®-11, a solid derivative of HyA, a naturally occurring component of the body.

Rats were selected as the experimental animal because they are inexpensive, widely available, acceptable to society, and easily housed and maintained[17]. Because of ethical concerns, the unilateral defect model was used in this study. Non-critical-sized defects were created because the selected materials were void fillers. The 7th and 21st days were selected for euthanasia to determine the healing process of the bone defects in relation to the methods used in previous studies[12,18].

Osteoconduction is the process that allows bone apposition from existing bone. Osteoconductive graft materials provide an environment that is capable of hosting the mesenchymal stem cells, osteoblasts, and osteoclasts that are essential for the functioning of the bone graft[1]. Collins et al. showed that there was no significant difference in the new bone formation after the implantation of biphasic calcium sulfate alone or in combination with gamma-radiated human mineralized allografts in the extraction sockets[19]. In a comparison of three bone grafting materials, Zhang et al. found that biphasic calcium phosphate bone grafts resulted in a greater amount of new bone formation than did demineralized freeze-dried bone allografts and natural bone minerals of bovine origin[20]. No complications were observed during the current experiment; therefore, Gr-MA can be safely used for bone formation.

HyA has been shown to play an important role in the healing of bone tissue[21]. Several studies have confirmed the efficacy of HyA on the soft tissues[22-25]. Studies have more recently focused on the effects of HyA on bone healing. Demonstrating higher bone volume, Huang et al. asserted that HyA, with an optimal combined administration, could significantly promote the osteogenic and angiogenic activity of bone morphogenic protein 2 (BMP-2) or absorbable collagen sponges (ACSs)[25]. Mermerkaya et al. demonstrated the role of hyaluronan-based mesh in promoting osteoblastic function[26]. In a comparison of HYAFF®-11 in vitro and HyA in various molecular weights, Moseley et al. observed that the HYAFF®-11 had superior wound healing and antioxidant capacity[27]. Mendes Brazão et al.[28] performed a radiological examination of a critical-sized defect in rat calvaria to examine the effects of HyA on bone healing. They concluded that HyA alone or in association with a carrier would not improve bone healing. This was consistent with the findings of the present study. Bezerra et al.[29] investigated the effects of HyA on bone healing in critical-sized calvarial defects in rats at 2 months post-surgery. They reported an increased amount of new bone formation in the defects filled with HyA gel and HyA gel + ACS. In the current study, the HyA group exhibited a lower amount of bone regeneration than the control group. This might have been related to the time appointment and the density of the HyA.

Bone graft materials can be applied with other kinds of materials to increase their effectiveness. HyA is used in combination with other graft materials to improve wound healing and bone regeneration[30]. Arpağ et al. investigated the effects of HyA, xenografts, and autografts on rabbit calvarial defects. They asserted that HyA contributes to xenograft in new bone formation and bone healing by reducing the residual graft volumes[31]. Koca et al. reported on the positive contributions of HyA alone or in combination with grafts to healing in critical-sized bone defects in rat jaws[32].

Unlike the abovementioned studies, the current study found that HyA alone did not have an effect on new bone formation or the healing scores. Diker et al.[33] found that HyA did not adequately improve bone regeneration in rats. However, they reported that bone formation was more noticeable in the graft and HyA + graft defects. Agrali et al. asserted that HyA alone or in combination with a graft and membrane did not significantly contribute to bone regeneration in critical-sized rat calvarial defects[34]. The current study obtained similar results.

The contribution of osteoconductive materials is not affected by the size of the defect; thus, a critical-sized defect was not chosen for the current study. The goal was to determine the effectiveness of the materials used as void fillers. The study used rat tibial defects; however, the findings of previous studies were not supported. HyA alone or in combination with grafts did not improve the healing scores.

Aslan et al.[35] stated that the amount of the bone formation in the rat tibial defects filled with HyA + allogenic cancellous bone grafts was greater than that in defects filled with allogenic cancellous bone grafts

only. In contrast, the current study found no significant differences in new bone formation in the Gr-MA, HyA, and Gr-MA + HyA groups. One of the reason could be the duration of the different postoperative healing periods in the studies. In our study we only showed the early healing scores, although Aslan et al. analyzed the the 40th postoperative day of healing. The other reason could be the properties of the HyA used in the study. It had a more stable structure than the equivalents produced in liquid form, it took longer to biodegrade, and it acted as a place holder by turning into a gel after coming into contact with blood. The current study was planned on the basis of the expected effects of the HyA. It is possible that the consideration of the expected effects of the bone graft would have produced different results.

The present study has several limitations. First, the small number of animals limited the number of samples that could be obtained. Second, undecalcified sections, which could have strengthened the study results, were not used. Third, conducting the study over three experiment periods could have facilitated the assessment of the effectiveness of the use of HyA exclusively or in combination with graft materials during the recovery.

As a conclusion, hyaluronic acid has a versatile role in tissue repair process from early-stage inflammatory activity to tissue generation granulation. Its effects on process of both soft tissue healing and wound healing are shown in many studies. Within the limitations of the present study, it can be suggested that HyA, used alone or in combination with Gr-MA, is not likely to enhance bone formation in rat tibial defects. There is still much work needed to demonstrate the effects and biological mechanisms of hyaluronic acid in bone healing.

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