Original Article

Braz J Oral Sci.
April/June 2010 - Volume 9, Number 2

Hyaluronan does not improve bone healing in critical size calvarial defects in rats - a radiographic evaluation

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Abstract

Aim: This study evaluated radiographically the effects of 1% hyaluronan in bone healing using a critical size rat-calvaria defect model. **Methods:** Thirty adult male Wistar rats were used in this study. Two 6-mm-diameter critical-size defects were created and the treatments were randomly distributed as follows: **1)** 1% hyaluronan; **2)** 1% hyaluronan soak loaded onto an absorbable collagen sponge (ACS) carrier; **3)** saline; and **4)** ACS alone. The animals were sacrificed at 4 and 8 weeks when biopsies were collected and radiographs obtained using a direct digital radiograph system and a standardized protocol. A blind examiner evaluated the radiographic density of the images twice and an intraclass correlation was performed to evaluate examiner reproducibility (R2=0.99, p<0.001). Comparisons between 4 and 8 weeks of treatment were performed by Student's t test and comparisons between treatments and time by two-way ANOVA at 5% significance level. **Results:** There were no noteworthy differences between 4 or 8 weeks within each treatment group (p>0.05). When treatments were compared no significant differences between groups were found (p>0.05). **Conclusions:** Within the limits of this study, it can be concluded that 1% hyaluronan gel alone or its association with a carrier does not improve bone healing.

Keywords: bone repair, wound healing, hyaluronan.

Introduction

Hyaluronic acid (HA) is a high molecular weight polysaccharide ubiquitously distributed in the extracellular space of higher animals; the highest concentrations are found in soft connective tissues. In recent years, HA has been reported to play critical roles in a wide variety of biological events, such as wound healing, chondrogenesis, osteogenesis, the immune response, and migration of rat transformed cells[1-3].

Sasaki and Watanabe[4] showed that HA is capable of accelerating new bone formation through mesenchymal cell differentiation, in bone created wounds in the animal model. They were able to demonstrate that bone formation had already been induced at day 4 after the application of HA. HA possesses biochemical and physical properties suitable to perform an important role in the early events of

Received for publication: March 02, 2010
Accepted: June 01, 2010

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osteogenesis as well as in many other tissues. It is a prominent extracellular matrix component during bone morphogenesis[5] and large amounts of HA are present during the transition from mesenchymal cell to cartilage[6]. In terms of its correlations with wound healing mechanisms and hard tissue development, HA can be thought as a "primer" in cell regeneration.

Bone maintenance and bone regeneration have become essential concepts in the treatment of periodontal disease, in the healing of tooth extraction sockets and in the utilization of dental implants. Currently, much effort has been made not only to maintain and prevent bone loss, but also to augment and regenerate bone around teeth and implants and to rebuild edentulous ridges.

These findings suggest that associating HA with devices, such as absorbable collagen sponges and membranes, may help improve bone regeneration. This study evaluated radiographically the effects of 1% hyaluronan in bone healing using a critical size rat-calvaria defect model.

Material and methods

Thirty 12-week-old male Wistar rats with mean body weight of 300 g. were used in this study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimate to the laboratory environment for a 5-day period. The protocol was approved by the State University of Campinas Institutional Animal Care and Use Committee under the protocol #1112-1.

General anesthesia was obtained by intramuscular administration of a combination of ketamine chloride (50 mg/kg) and xylazine chloride (15 mg/kg). The surgical site was shaved and washed with iodine. An L-shaped incision was made and a full-thickness flap including periosteum was reflected, exposing the calvaria bone.

In each animal, two 6-diameter round defects were created, one on each side of the mid-sagittal suture, with a trephine bur in a dental handpiece under constant irrigation of sterile saline. The trephined bone was removed from the surgical field.

Each animal was included in two different treatments. Four treatments were evaluated: 1) 1% hyaluronan gel (HA); 2) 1% hyaluronan soak-loaded onto an absorbable collagen sponge (ACS) carrier; 3) saline; and 4) ACS alone. Care was taken to have both hyaluronan groups in the same animal so that no carry-over of the gel would occur between defects, avoiding bias to the results. The periosteum and skin were sutured for total coverage with 5-0 nylon suture. This way, 15 animals were assigned for the 4 week time point and another 15 for the 8 week time point. There were 8 animals in treatment groups 1 and 2, and 7 animals treatment groups 3 and 4 at each time point. Animals were euthanized by an overdose of anesthesia.

Radiographs were taken using the direct digital radiograph system Sens-A-Ray 2000 (Regan Medical Systems AB, Sundsvall, Sweden) and a dental radiograph unit (Dabi Atlante, Ribeirão Preto, Brazil) (70kVp, 7mA, 0.1s) (Figure 1). The distance between the x-ray source and the digital sensor was 20 mm. The relative bone density was measured as described by Ahn et al.[7]. Defect radiodensity was assessed by ImageJ (version 1.38, National Institutes of Health, Bethesda, MD, USA).

Intra-examiner reproducibility was examined by repeated evaluation of all defect sites ($R2 = 0.99$, $p < 0.001$). Comparisons between 4 and 8 weeks of treatment were performed by Student's t test and comparisons between treatments and time by two-way ANOVA. The level of significance was set at 5%.

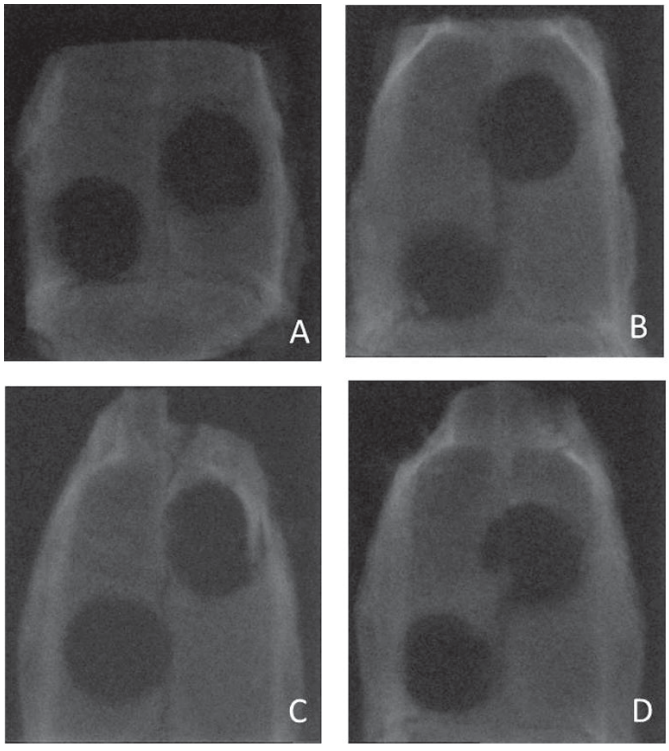

Fig. 1. Radiographs showing calvarial defects in each treatment group. A- ACS (left defect), Saline (right defect) 4 weeks; B- ACS (left defect), Saline (right defect) 8 weeks; C- HA+ACS (left defect), HA (right defect) 4 weeks; D- HA+ACS (left defect), HA (right defect) 8 weeks.

Results

The results of the radiographic analysis are shown in Table 1. At 4 weeks post-surgery mean radiodensity (±SD) for saline, ACS alone, 1% hyaluronan alone and 1% hyaluronan/ACS amounted to 13.36 ± 2.67%, 13.86 ± 2.79%, 14.41 ± 3.50%, and 16.69 ± 3.12%, respectively, with no significant differences among treatments ($p > 0.05$). At 8 weeks post-surgery the corresponding values were 12.79 ± 5.04%, 13.26 ± 2.91%, 11.40 ± 1.94%, and 13.99 ± 3.16%, respectively, and no statistically significant differences among treatments were observed ($p > 0.05$). No statistically significant interaction effects were found between treatment and time ($p > 0.05$).

Discussion

The aim of this study was to evaluate the effects of hyaluronan on bone healing in a critical size calvaria defect

Table 1. Relative bone density (mean ± sd) of treatment groups according to time.

	4 weeks	8 weeks
Saline (n=7)	13.36±2.67^{Aa}	12.79±5.04 ^{Aa}
ACS (n=7)	13.86±2.79 ^{Aa}	13.26±2.91 ^{Aa}
HA (n=8)	14.41±3.50 ^{Aa}	11.40±1.94 ^{Aa}
HA+ACS (n=8)	16.69±3.12 ^{Aa}	13.99±3.16 ^{Aa}

Distinct uppercase letters in rows indicate statistically significant differences among treatments (Two-Way ANOVA, p<0.05). Distinct lowercase letters in columns indicate statistically significant differences between times (Student's t test; p<0.05).

model. The calvaria defect, compared to other experimental bone defects, is a convenient model for studying bone regenerative materials because of its effective accessibility and the lack of fixation requirements. Schmitz and Hollinger[8] suggested that an 8-mm-diameter defect is suitable to evaluate candidate biomaterials for bone regeneration and constitutes a critical-size defect in the rat. This experimental design has been utilized in previous studies to evaluate the osteoconductive/inductive potential of candidate biologics, biomaterials, and devices for bone reconstruction[9-,11]. Others have defined and successfully used smaller critical-size rat calvarial defects[12-13]. Thus, the geometric and physiological nature of the rat calvaria and the 6 mm trephine defects used in this study appear adequate to investigate the regenerative potential of hyaluronan.

Radiographic evaluations have been used to evaluate the biologic potential of various devices, as well as osteoconductive/inductive biomaterials and biologics to promote local bone formation in rat calvarial defects. Briefly, barrier membranes for guided bone regeneration[12-13], autograft bone, and growth and differentiation factors, including purified protein constructs[9-11] or combinations thereof, have been evaluated.

The radiographic observations in these studies demonstrated limited radiographic bone fill in the control sites. This is in agreement with the findings in this study where complete defect closure was not observed.

The results demonstrate that radiographic evaluations of bone formation are associated with significant weaknesses, and, as such, poorly represent actual healing events. Radiographic evaluations have been used to evaluate the effect of various treatment concepts on bone formation. It appears from the present study and from other investigations[7,14] that radiographic analyses should be viewed with caution, and that observations of bone healing in experimental models such as herein or in more complex models should be confirmed using histologic observations[13-16]. A histological evaluation will be necessary to verify the real effects of HA on bone healing in this experimental model.

A greater evaluation period may also be necessary in this model to observe significant differences when conducting a radiographic evaluation, however, some of the studies which use this model last 8 weeks and they show good results. The only difference is that they perform a qualitative evaluation[17-18] or use more sensitive radiograph methods such as micro-ct scans[4,19]. Pryor et al.[14] pointed out that bone healing in animal models aiming at treatment recommendations for clinical application must not only be determined based on radiographic analysis, but should also be confirmed histologically.

Previous studies have also attempted to establish HA as an alternative in bone reconstruction. HA was used as raw material for hydrogels, sponges and polymers. In order to increase the bone regenerative effects growth factors, mesenchymal stem cells and demineralized bone matrix have been added to this material[20-21]. Due to the natural presence of HA as major part of the extracellular matrix of many tissues this seemed to be a promising approach.

Aslan et al.[22] examined the effect of autologous bone graft versus autologous bone graft and HA in a defect model in rabbit tibiae. They documented that HA needs an osteoconductive scaffold to be effective because the addition of HA leads to higher scores for bone formation in each time period of the study. Wiedmann-Al-Ahmad et al.[23] documented one of the highest proliferation rates for HA when compared to 16 different biomaterials. Kim et al.[21] showed that a hyaluronic-based hydrogel is suitable as carrier for human mesenchymal stem cells and BMP-2.

Another explanation for the present results may be that HA did not have the optimal molecular weight. Different authors examined the effects of various molecular weights on osteogenesis. Pilloni and Bernard[24] showed that low molecular weight HA accelerated osteogenesis in vitro in a bone marrow ablation model in rat. In contrast to this study, Sasaki and Watanabe[4] described that high molecular HA increases bone formation after bone marrow ablation in comparison to untreated controls. The results of the studies about the optimal molecular weight are therefore inconsistent at best. Histomorphometric analyses of the specimens are underway to better understand the healing dynamics of these defects as a result of each treatment evaluated.

Within the limits of this study, it may be concluded that 1% hyaluronan gel alone or its association with a carrier (ACS) was not able to improve bone healing in this experimental model.

Acknowledgements

Dr. Brazão received financial support by The National Council for Scientific and Technological Development (CNPq), Brasilia, DF, Brazil.

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