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**Original Article**

# Hyaluronan Enhances Cranial Bone Healing in Rats

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*Hyaluronan (HA) is one of the major glycosaminoglycans in bone and connective tissue, and enhances wound healing. The purpose of this study was to evaluate the usefulness of HA in the healing of cranial bone defect in rats. A hole (1.5 mm in diameter) was drilled in the cranial bone of male Wistar rats. HA solution or phosphate-buffered saline was then applied to the bone defect. Bone sections were stained histochemically and immunohistochemically. The newly formed bone area was quantified microscopically. On day 21, the mean area of newly formed bone in the HA group was significantly larger than in the control group. The defect closure (%) was significantly higher on days 14 and 21 in the HA group than in the control group. Stronger staining for alkaline phosphatase was noted in the area adjacent to the defective bone in the HA group than in the control group on days 7 and 14. On day 14, strong immunoreactivity for type-1 collagen was identified in the defect of the HA group compared to the control group. Our results suggest that HA enhances the cranial bone healing process in rats, suggesting it could be useful for alveolar bone regeneration.*

Key words: Hyaluronan, Cranial bone, Bone defect, Rat

## INTRODUCTION

Hyaluronan (HA) is a natural tissue component known to play an important role in the function of extracellular matrices, such as that of the periodontium[1-3]. We have reported that HA is localized in gingival connective tissue and oral epithelium by immunohistochemistry using a specific binding protein; HA binding protein[4]. In this regard, the localization of HA changes during inflammation, as demonstrated in experimentally induced periodontitis in dogs[4]. HA also plays a regulatory role in the processes of inflammation and wound healing[5]. The application of exogenous HA and HA-based biomaterials has been successful in manipulating and accelerating the wound healing process in a number of medical disciplines, including ophthalmology, dermatology and rheumatology[5,6].

Periodontal tissue is constructed of epithelium, connective tissue, and bone. One focus of treatment of the periodontal defect is the regeneration of bone and new attachment of the periodontal ligament and soft tissue. The purpose of this study was to test the effects of HA on the healing of cranial bone defects in rats.

## MATERIALS AND METHODS

Experiments were conducted in 30 male Wistar rats (4 weeks old). The rats were anesthetized with Nembutal (Abbott Laboratories, Chicago, IL). The scalp was cut in the center to expose the cranial bone. The latter was drilled with a dental round burr (1.5mm in diameter), taking utmost care in avoiding injury to the dura matter and brain tissue[7]. Then, 0.1m*l* (0.2% in phosphate-buffered saline [PBS], pH 7.4) of HA solution (Seikagakukogyo, Tokyo, Japan) or 0.1m*l* of PBS in control rats, was added to the bone defect. The skin was subsequently sutured with a surgical stapler and the rats were allowed to recover. Two or 3 weeks after surgery, the rats were sacrificed with deep anesthesia, and the heads were removed immediately and fixed with 10% of paraformaldehyde containing PBS for 48 h. The experimental sites were carefully dissected out and removed. The tissues were decalcified with 5% ethylenediaminetetraacetic acid (EDTA) solution for 3 weeks at room temperature. The tissues were embedded in paraffin and cut serially. The sections were stained with hematoxylin and eosin (H&E). The prepared slides were analyzed histomorphometrically using a Video Micro Meter (VM-30; Olympus, Tokyo) to measure the newly formed bone area, the distance between the defective margins, and the distance between new bone growth points. The defect closure (%) represented the distance between the edges of newly formed bone divided by the distance between the defective margins x100.

Other sections were stained for alkaline phosphatase activity with NTB/BCIP (Boehringer Mannheim, Mannheim, Germany) after treatment with 50 nM $MgCl_2$ containing 0.1 nM Tris buffer (pH 9.5) for 12 h. In addition, sections were stained immunohistochemically using anti-type 1 collagen polyclonal antibody (LSL, Tokyo), and biotin conjugated hyaluronan-binding protein (Seikagakukogyo, Tokyo).

The histomorphometric data were expressed as the mean ± SD. Differences between HA and control groups were tested for statistical significance by the Mann Whitney *u*-test. P value < 0.05 was significant.

All experiments were approved by the Animal Care Committee of School of Dentistry, Asahi University.

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## RESULTS

*Histological changes*

Histological examination on day 7 showed numerous inflammatory cells and erythrocytes at the experimental site in the control group; however, fewer inflammatory cells and increased numbers of fibroblasts were seen at the site in the HA group. On day 14, new bone formation adjacent to the edge of the original bone was noted in the HA group, and osteoblasts lined the surface of the newly formed bone (Fig. 1 b). In the control group, minimal bone formation was seen and only in limited areas (Fig. 1 a). On day 21, the experimentally created bone defect was almost filled with newly formed bone from both sides of the original bone in the HA group (Fig. 2 b); however, new bone formation was incomplete in the control group (Fig. 2 a).

*Changes in ALP activity*

On day 7, strong staining for ALP was noted on the surface of newly formed bone in the HA group; however, such activity was relatively less pronounced in the control group. On day 14, ALP activity was stronger in the HA group than in the control group. On day 21, ALP activity was weaker in this area in the HA group but not in the control group.

*Histomorphometric changes*

The gap between the margins of the created defect in the HA group and control group was similar on days 7, 14, and 21 (Fig. 1). All sections showed dissection at the center of the defect area (Fig. 1, 2). The newly formed bone area was quantified under a microscope. On day 21, the area of newly formed bone in the HA group ($2.69 \pm 0.63 \times 10^5 \mu m^2$) was significantly larger than in the control group ($1.56 \pm 0.23 \times 10^5 \mu m^2$) (n=5 each, $p<0.01$) (Table 1). The defect closure (%) on day 14 in the HA group ($53.3 \pm 6.7\%$) was significantly higher than in the control group ($24.7 \pm 3.2\%$). Similar findings were noted on day 21 (HA: $72.2 \pm 11.2\%$, Control: $30.7 \pm 4.1\%$, n=5 each, $p<0.01$).

Stronger staining for ALP was noted in the area adjacent to the defective bone in the HA group than in the control group on days 7 and 14. Furthermore, on day 14, stronger immunoreactivity for type-1 collagen was identified in the defect area in the HA group compared with the control group.

Table 1 Histomorphometric changes

| | Distance between original bone edges | Area of new bone | Area of the defect closure (%) |
|---|---|---|---|
| **Day 7** | | | |
| HA | 1.53±0.07mm | | |
| Control | 1.53±0.04mm | | |
| SD | NS | | |
| **Day 14** | | | |
| HA | 1.51±0.04mm | $9.98 \pm 1.99 \times 10^4 \mu m^2$ | 53.3 ± 6,7% |
| Control | 1.51±0.03mm | $4.79 \pm 1.88 \times 10^4 \mu m^2$ | 24.7 ± 3.2% |
| SD | NS | p<0.05 | p <0.01 |
| **Day 21** | | | |
| HA | 1.57±0.07mm | $2.69 \pm 0.63 \times 10^5 \mu m^2$ | 72.2±11.2% |
| Control | 1.53±0.06mm | $1.56 \pm 0.23 \times 10^5 \mu m^2$ | 30.7±4.1% |
| SD | NS | p <0.01 | p <0.01 |

Histomorphometric changes between groups

n=5, SD: significant difference, NS: not significant

The diameter of the cranial bone defect between groups was not significant. Area of new bone in the HA-treated group was higher than in the control group. The defect closure(%) in the HA−treated group was higher than in the control group.

Mean±SD $P>0.01$ $p<0.05$: significant difference(SD) HA vs control(n=5).

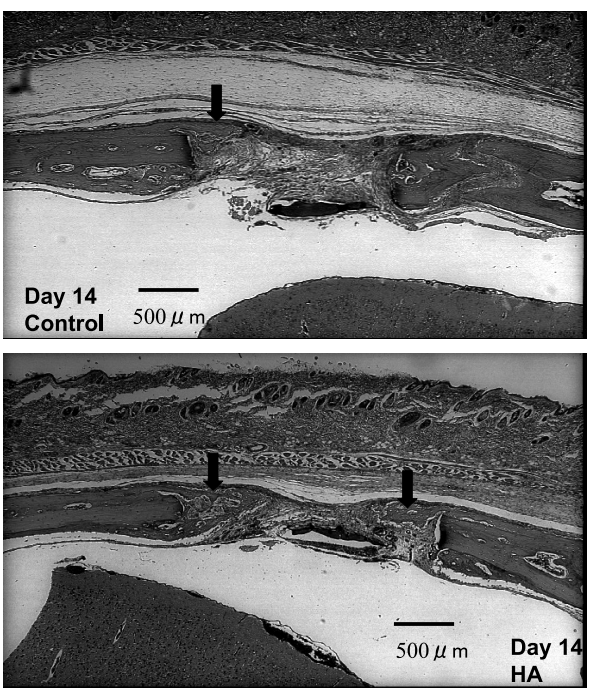


Fig. 1 a. Newly formed bone in control 14 days after surgery. Arrow indicates new bone formation.

Fig. 1 b. Newly formed bone in HA-treated bone defect 14 days after surgery. The area of new bone was higher than in the control. Arrows indicates new bone formation.

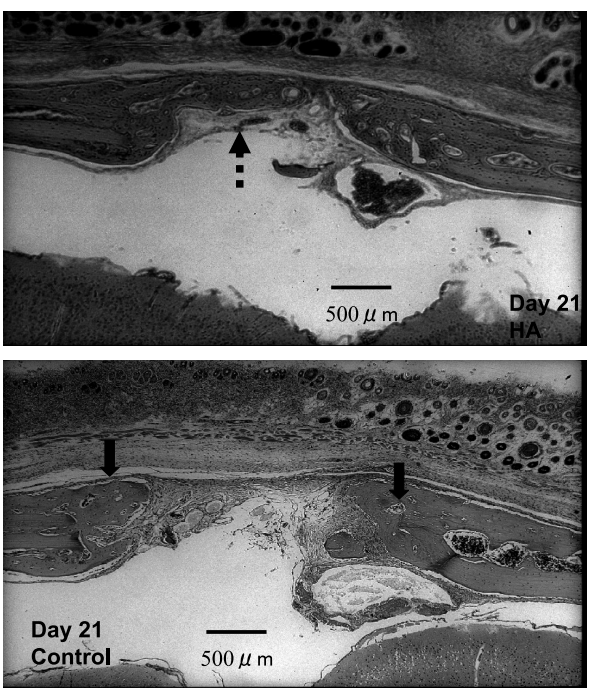


Fig. 2 a. Cranial bone in control 21 days after surgery. The defect has not recovered yet. Arrow indicates new bone.

Fig. 2 b. Cranial bone in HA-treated bone defect 21 days after surgery. The bone defect has been filled with new bone. Arrow indicates new bone formation.

## DISCUSSION

Hyaluronic acid(HA) is the most abundant high molecular weight glycosaminoglycan in the extracellular matrix of soft periodontal tissues[8,9]. This molecule is also important in relation to the mechanisms associated with inflammation and wound healing. The application of exogenous HA and HA-based biomaterial has been successful in manipulating and accelerating the wound healing process in a number of medical disciplines, including ophthalmology, dermatology and rheumatology[10].

In this study, we employed the calvarial bone defect model to test the effects of HA on bone formation because the bone healing process of this bone defect model is probably simple and numerous studies have used the model to investigate the bone healing process[7].

The results of histological examination suggest that HA enhances the cranial bone healing process. HA decreased the inflammatory reaction and enhanced fibroblast proliferation and osteoclastic activity following bone formation[5-8]. These results suggest that HA enhances a rapid response to injury turnover and modulates bone regeneration. Our results also showed increased ALP activity at the experimental site, indicating increased osteoblastic activity and associated bone formation activity.

The distance between the margins of the created defect was almost equal in all sections. The selected defect size of 1.5mm was based on preliminary studies, which showed complete closure of the created defect by day 21. The present study was not designed to investigate the role of HA on unexpected bone formation, but the speed of bone formation in a limited area, compared with the control. The results showed that bone formation activity on days 7 and 14 in the HA-treated group was higher than in the control group, although the control group had achieved almost complete closure of the bone defect by day 21.

HA plays a role in embryogenesis, wound repair, and regeneration. The initial healing events include bleeding and clotting followed by secretion of collagenase and HA. The immediate presence of collagenase and HA influences cell-to-cell contact so that cells may not readily adhere to matrix components, such as collagen, but rather proliferate, increase in density, and migrate. In addition, HA may prevent or delay lymphocytes, macrophages, and neutrophils from entering the wound healing sites, thus providing protection against proteolytic digestion by inflammatory cells. High molecular weight HA may enhance cell migration while low molecular weight HA may enhance mitosis. HA fragments also sometimes exhibit angiogenic activities. HA may prevent wound dehydration due to its structural ability to sequester water. HA degrades in the absence of inflammatory reactions. Furthermore, HA has been shown to have bacteriostatic activity, and HA biomaterials have been shown to be functional carriers of other biologics and cells[11-13].

Based on the present results, it is conceivable that hyaluronan administration to periodontal bone defect could achieve comparable beneficial effects in periodontal healing and surgery, hence aiding the treatment of periodontal disease[10].

In conclusion, the present study suggested that HA enhances cranial bone healing process in rats, suggesting that HA could be useful for alveolar bone regeneration.

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# ラット頭蓋骨骨欠損におけるヒアルロン酸投与の影響

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ヒアルロン酸は主要グリコサミノグリカンであり，結合組織の代謝や創傷治癒に重要な役割を担っている．本研究の目的は，ヒアルロン酸の骨再生への影響を検討するために，ラット頭蓋骨の人工的骨欠損部位へのヒアルロン酸（HA）と治癒過程に及ぼす影響を組織学的に検討することである．

ウイスター系雄性ラットの頭部にダイヤモンドバーを用いて人工的に頭蓋骨に骨欠損を作製した．コントロール群にはリン酸塩緩衝液を，実験群はHA溶液を塗布した．術後7，14，21日目で屠殺し組織切片とした．組織染色はHE染色，ALP染色を行った．組織形態計測として，既存骨切除面間距離，新生骨形成量，骨閉鎖率（％）を計測した．

HE染色像において，術後７日目では各群において明らかな差違は見られなかった．術後14日のHE染色像においてHA溶液塗布群で新生骨の形成が他の群に比較して増加していた．術後21日のHE染色像においてもHA溶液塗布群で新生骨形成量がコントロール群に比較して多かった．アルカリフォスファターゼ染色においてはHA溶液塗布群がその他の群に対して治癒の過程の早期に高いALP活性が見られた．

組織形態計測の結果，新生骨形成量は21日目でHA群が有意に高かった．骨閉鎖率は14日目，21日目ともにHA溶液塗布群においてコントロール群と比較すると有意な差が認められた．Ⅰ型コラーゲン抗体による免疫組織染色において14日目に強い陽性反応がみられた．

以上のことより，骨欠損部にHA溶液を塗布することにより，骨欠損部の骨修復を促進することが示唆された．

キーワード：キーワード：ヒアルロン酸，ラット頭蓋骨，人工的骨欠損

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