치의과학박사 학위논문

Comparative Evaluation of the Optimal Dosage of Hyaluronic Acid for Bone Regeneration in Rat Calvarial Defects

랫드 두개골 결손부 골재생을 위한 적정 히알루론산 투여량에 대한 비교 평가

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Comparative Evaluation of the Optimal Dosage of Hyaluronic Acid for Bone Regeneration in Rat Calvarial Defects

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Abstract

Comparative Evaluation of the Optimal Dosage of Hyaluronic Acid for Bone Regeneration in Rat Calvarial Defects

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Purpose:

Hyaluronic acid (HA) affects angiogenesis and promotes the migration and differentiation of mesenchymal cells, thereby activating the osteogenic ability of osteoblasts. Although studies on the action of HA during bone regeneration are being actively conducted, the optimal dose of HA required for bone regeneration remains unclear. Therefore, the purpose of this study was to elucidate the most effective HA dose for bone formation using a rat critical-size defect model.

Methods:

Thirty rats were randomly divided into 5 groups, with 6 rats in each group. An absorbable collagen sponge soaked with HA or saline was used to fill an 8-mm defect, which was then covered with a collagen membrane. Different treatments were performed for each group as follows: (1) saline control, (2) 1 mg/mL HA, (3)

25 mg/mL HA, (4) 50 mg/mL HA, or (5) 75 mg/mL HA. After a healing period of 4 weeks, micro-computed tomography and histological analysis were performed. The obtained values were analyzed using analysis of variance and the Tukey test (P<0.05).

Results:

At week 4, the 75 mg/mL HA group had the highest BV/TV, new bone, and BVA among the five groups. The 75 mg/mL HA group was significantly different from the values observed in the control group and the 1 mg/mL HA group for BV/TV, NB, and bone fill, and was significantly different from the control group, 1 mg/mL HA group and 25mg/mL HA group for BVA. More active bone formation was observed in the higher-dose HA groups (25 mg/mL, 50 mg/mL, and 75 mg/mL HA), which included a large amount of woven bone.

Conclusions:

The bone formation in the 75 mg/mL HA group was superior to the other groups (1, 25, and 50 mg/mL HA and control), thus demonstrating the dose-dependent effect of hyaluronic acid in bone regeneration.

Keyword : Animal model; Bone; Bone regeneration; Hyaluronic acid; Rats

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Chapter 1. Introduction

Bone regeneration has become a fundamental concept of dental treatment and is widely used for the restoration of periodontal defects, dental implant therapy, and dental prosthetic treatments.[1] Currently, guided bone regeneration (GBR) therapy is carried out using bone graft materials, barrier membranes, and biologics, and promising bone regeneration results have been obtained.[2] Due to the different materials used in GBR, the bone regeneration capacity varies substantially. Therefore, research is needed to find better substances for promoting bone formation and maturation.

Hyaluronic acid (HA), also called hyaluronan or hyaluronate, is present in the majority of mammalian tissues, including the soft connective tissue, synovial fluid of the joints, the lung, kidney, brain, and muscle tissue.[3,4] As a glycosaminoglycan, HA is a crucial component of the extracellular matrix that is necessary for a variety of biological functions and plays an essential role in wound healing.[5,6] During the early bone regeneration process, fibroblasts secrete HA.[7] Previous studies have shown that HA is involved in cellular processes, such as morphogenesis, wound healing, inflammation, and metastasis.[5,8-11] Additionally, HA has been shown to affect angiogenesis and can enhance the osteogenic capacity of osteoblasts by promoting the migration and differentiation of mesenchymal cells.[12-14] HA has low immunogenicity and high biocompatibility, making it especially useful compared to other growth factors or scaffolds used during bone healing.[15,16] These advantages have drawn attention to the potential of HA as an excellent bone regeneration material.

In recent years, research on the impact of HA on the bone healing process has demonstrated its promise. Studies conducted in both normal and infected sockets showed that HA promotes bone formation and wound healing.[17,18] A study by Huang et al.[19] showed that HA had dose-dependent and molecular weight-specific effects on cell proliferation, differentiation, and bone formation, particularly at high molecular weights, where alkaline phosphatase activity significantly increased.[19] However, there is an absence of research on the ideal HA dose required for bone healing *in vivo*. Determining the HA dose required for bone formation and maturation would be enormously helpful for the GBR process.

Hence, the aim of this study is to explore the optimal dose of HA for bone regeneration using a rat critical-size defect model. The null hypothesis in this study was that HA soak-loaded in absorbable collagen sponge (ACS) does not enhance regeneration as compared to spontaneous healing in bone defects.

Chapter 2. Materials and methods

Animals

All research protocols were approved by the Institutional Animal Care and Use Committee, Seoul National University, Korea (SNU-190722-2-2). Thirty 11- to 13-week-old male Sprague–Dawley rats (OrientBio, Seoul, Korea) weighing 300-400 g were used for this experiment. The rats were acclimatized for at least 1 week before the experiments. The animals were randomized into 5 groups, each containing 6 animals. Each plastic cage housed two animals with tail-marking in the monitoring environment (temperature 23°C; humidity controlled at 60%; 12:12-hour light-dark cycle). They had *ad libitum* access to water and a standard pellet rat-food diet. All experiments were performed according to the guidelines of the Institute of Laboratory Animal Resources, Seoul National University.

Formulations of hyaluronic acid

The different doses of HA used in this study were obtained by mixing HA with a molecular weight of 1000 kDa (Shiseido Sodium Hyaluronate SZE [Grade-EP], Shizuoka, Japan) and phosphate-buffered saline. The concentrations were as follows: 1 mg/mL, 25 mg/mL, 50 mg/mL, and 75 mg/mL. After mixing, the HA was sterilized at 121°C for 15 minutes.

Surgical procedures

The animals were anesthetized by an intraperitoneal injection of a combination of 25 mg/kg tiletamine/zolazepam (Zoletil 50; Virbac, Carros, France) and 10 mg/kg

xylazine hydrochloride (Rompun; Bayer Korea, Ansan, Korea). After anesthesia, the dorsal surface of the head was shaved and disinfected with povidone-iodine. Routine invasive anesthesia was performed at the surgical site with 2% lidocaine HCl and epinephrine (1:100,000; Huons, Seongnam, Korea). A 3-cm midline incision was made through the skin along the sagittal suture of the skull, and the soft tissues and periostea were elevated and reflected (Figure 1A). Under saline irrigation, craniotomy defects with an 8-mm diameter were created in rats using a trephine bur (Figure 1B). An ACS (Collagen Graft 2; Genoss, Suwon, Korea) cut into the shape of the defect with a diameter of 8 mm was prepared in advance (Figure 1C). The rats were divided into 5 groups as follows: (1) control group, the defect area received an ACS soaked with saline; (2) 1 mg/mL HA group, the defect area received an ACS soaked with 1 mg/mL HA; (3) 25 mg/mL HA group, the defect area received an ACS soaked with 25 mg/mL HA; (4) 50 mg/mL HA group, the defect area received an ACS soaked with 50 mg/mL HA; (5) 75 mg/mL HA group, the defect area received an ACS soaked with 75 mg/mL HA. The collagen soaked in HA or saline was placed in the defect site (Figure 1C and D), and a collagen membrane (Collagen Membrane-P; Genoss, Suwon, Korea) was used to cover the surgical site (Figure 1E). The incision was sutured in layers with 4-0 Vicryl (Ethicon, Raritan, NJ, USA) and 5-0 Monosyn (B. Braun Surgical, SA Rubi, Spain) (Figure 1F). All rats received intramuscular injections of antibiotics (cefazolin; Chong Kun Dang, Seoul, Korea) and analgesics (Meloxicam; Labina, Barcelona, Spain) directly after surgery. Four weeks after surgery, the rats were sacrificed with excess CO_2 gas.

Micro-computed tomography

Tissues, including the surgical sites, were harvested and fixed in 10% neutralized buffered formalin, and micro-computed tomography (micro-CT) images were taken using a micro-CT device (SkyScan 1173; Bruker, Kontich, Belgium) at a pixel size of 9.94 μm (130 kV, 60 μA). Micro-CT data were measured 3 times by a single blinded and calibrated examiner (LL) using the same software (CTan; Bruker, Kontich, Belgium). Three-dimensional visualization images were obtained using CTVox software (Bruker, Kontich, Belgium). The bone volume/total volume ratio (BV/TV) was measured for each defect with a gray threshold level of 52-250. In the defect field, a cylindrical area with a thickness of 0.7 mm from the base along the original defect margin and a diameter of 8 mm was defined as the volume of interest.

Histological preparation and analysis

The specimens were decalcified with a 10% EDTA solution for 2 weeks before they were dehydrated by a series of ethanol solutions of increasing concentrations and embedded in paraffin. A coronal section with a thickness of 5 μm through the center of the circular defect was obtained and stained with hematoxylin and eosin. The prepared specimens were examined by light microscopy. After a microscopic examination, a photograph of each slide was taken using a digital slide scanner (PANNORAMIC 250 Flash III; 3DHISTECH, Budapest, Hungary), and the resulting images were saved on a computer for analysis. A single blinded, calibrated examiner examined all of the images 3 times using the same software (ImageJ 1.53e; National Institutes of Health, Bethesda, MD, USA). The following

parameters were recorded for each defect site:

- Defect area (DA): the total defect area was defined as the area surrounded by 2 defect margins and 2 phantom lines drawn along the inner and outer calvarial bone contours;
- New bone area (NB): the total area of newly formed bone in the defect area;
- Bone fill: the fraction (%) of newly formed bone within the defect area.
- Blood vessel area (BVA): the total area of blood vessels in the defect area

Statistical analysis

The sample size was calculated based on a previous study considering 2% of new bone formation as clinically relevant, with a standard deviation of 1% and allocating six animals in each group (α=0.05, and β=0.2).[20] All data are expressed as the mean ± standard deviation. Statistical analyses were performed using statistical software (SPSS version 25; IBM, Armonk, NY, USA). One-way ANOVA followed by the Tukey post-hoc test was used to determine the significance of mean differences between groups. A *P*-value less than 0.05 was considered to indicate statistical significance.

Chapter 3. Results

All 30 defect sites healed well with no significant signs of infection, inflammation, or postoperative bleeding. No noteworthy events occurred during the experiments.

Micro-CT analysis and morphometric evaluation

Figure 2 shows the BV/TV results obtained from micro-CT of the calvaria. The BV/TV values were 19.73±11.25 in the control group, 14.63±9.81 in the 1 mg/mL HA group, 30.22±8.27 in the 25 mg/mL HA group, 25.69±10.96 in the 50 mg/mL HA group and 40.03±13.88 in the 75 mg/mL HA group. The 75 mg/mL HA group had a significantly higher BV/TV than the control group ($P<0.05$) and the 1 mg/mL HA group ($P<0.01$) (Figure 3). The BV/TV values also generally tended to increase with increasing HA doses (Figure 3). In the micro-CT analyses, it could be observed that the defects in all the groups were partially closed (Figure 2). The greatest amount of new bone formation was observed in the 75 mg/mL HA group (Figure 2E, J and O). New bone formation around the defect margin was denser and thicker than that in the middle part of the defect.

Histological observations

Figure 4 shows histological images 4 weeks after defect creation. There was no sign of inflammation or infection in any group. The defect sites in each group were partially filled with mineralized bone, and the collagen membrane and ACS residues were not completely absorbed. More active bone formation was observed in the relatively high-dose HA groups (25 mg/mL, 50 mg/mL, 75 mg/mL HA

groups), and it could be seen that they contained a large amount of woven bone. Additionally, this newly formed bone was more apparent at the margins than at the center of the defects. Many blood vessels were observed in the 75 mg/mL HA group.

Histomorphometric analysis

The results of the histomorphometric analysis are shown in Table 1 and Figure 5. There were no statistically significant differences in defect areas among the 5 groups. The 1 mg/mL HA group had less NB and less bone fill than the control group, but without a statistically significant difference. The 25 mg/mL HA group and the 50 mg/mL HA group had similar NB and bone fill, which were both greater than those of the control group. However, the differences were not statistically significant. The 75 mg/mL HA group had the greatest NB and bone fill among the 5 groups, with values that were significantly higher than those of the control group ($P<0.01$) and 1 mg/mL HA group ($P<0.01$). The 75 mg/mL HA group showed more BVA than any other group, and significantly higher than control group ($P<0.001$), 1 mg/mL HA group ($P<0.01$), and 25mg/mL HA group ($P<0.01$).

Chapter 4. Discussion

This study was conducted to find the optimal dose of HA for bone regeneration. A critical defect size of 8 mm was used to analyze the appropriate HA dose, and a collagen membrane was used to increase the stability of the defect site. Four weeks after the critical defect was created in rat calvaria, the dose of 75 mg/mL showed the best bone healing compared to the other groups.

There is a close relationship between the dose of HA and cell proliferation and differentiation.[21] Previous *in vitro* studies have shown increased cell growth at higher HA doses.[19,22,23] However, Kaneko et al. observed that a high dose of HA strongly inhibited the development of mouse myoblastic cells and bone marrow cells into osteoblasts.[24] These results suggest that higher doses of HA are not necessarily better and that finding an appropriate threshold for the osteoinductive dose is very important in the process of bone healing. In the present study, the degree of bone regeneration varied with the dosage (Figure 2). Among the 5 groups used in the study (1, 25, 50, and 75 mg/mL HA, and control), the highest BV/TV was observed at 75 mg/mL. In addition, it was generally shown that with increasing doses of HA, newly formed bone also increased. A similar result was obtained from histological observations. In the histological evaluation, as the dosage of HA increased, more bone formation dispersed in a large area of the defect site was observed, especially in the 75 mg/mL HA group.

Histological analyses showed a large area of woven bone with more blood vessels

at higher HA doses (especially 75 mg/mL). This may have been influenced by the angiogenesis-promoting function of HA.[25] HA is indirectly involved in bone wound healing by stimulating angiogenesis.[7,26] Previous studies have also demonstrated the effect of HA on the differentiation of mesenchymal cells into osteoblasts.[19] Through these mechanisms, HA is believed to have a beneficial effect on improving the wound healing process.

Previous experiments conducted by Bezerra et al. showed that in 5-mm rat calvarial defects, the use of 1% HA gel and ACS resulted in more bone fill than ACS alone and blood clots.[1] However, when only 1% HA was used, bone formation did not improve.[1] In this study, there was no significant difference in bone formation in the control group compared with the 1, 25, or 50 mg/mL HA + ACS groups; the only significant difference was found for the 75 mg/mL HA group. This finding indicates that ACS is a suitable scaffold for bone formation. Additionally, if used with an appropriate dose of HA, a synergistic effect can be obtained during bone regeneration.

Other studies have used HA in combination with other biomaterials. Huang et al. reported that hyaluronic acid promotes osteogenic and angiogenetic activity when used in combination with bone morphogenetic protein 2 (BMP-2)/ACS.[27] In another study, a thiol-modified hyaluronan hydrogel showed better osteogenic capacity than ACS when used in combination with BMP-2.[28] In the above 2 experiments, the combined use of HA increased the osteogenic potential and reduced the side effects of BMP-2. This is due to the fact that HA enables the gradual, continuous release of BMP-2, which can promote bone growth over a

longer time.[27-29] In addition, several previous studies have demonstrated that using HA in conjunction with bone grafts can improve bone regeneration.[30-32]

In this study, Collagen Graft® 2 was used as an ACS, and this product is currently mainly used for soft tissue augmentation. This product, like ACS used in other bone regeneration studies[33,34], is made from pure collagen. The Collagen Graft® 2 is a two-layered structure in which a porous sponge layer is placed on top of a dense membrane layer. These structural features can support blood, have excellent space maintenance, and have stable decomposition characteristics. And in this study, we tried to observe the possibility of using Collagen Graft® 2 for bone regeneration. No inflammation or abnormal findings were observed in all rats treated with Collagen Graft® 2, and good bone healing was observed.

The significance of this study is that different concentrations of HA were specifically prepared to perform the experiments. Most current studies used commercially available HA products, so these studies either did not mention HA concentration or dosage or used a single concentration.[34,35] To the authors' knowledge, few studies have yet attempted this, although this information would greatly contribute to the study of the relevance of HA to bone regeneration.[36] However, the present work has several flaws, chief among them the inability to quantify the amount of HA and saline soaked into the ACS. HA may be lost due to suction or spread to other sites during surgery, which may have had a certain impact on the experimental results. Additionally, the effect of a higher dose of HA than 75 mg/mL on bone healing requires research. As the concentration of HA increases, its physical properties change. Preparing HA above 75mg/mL makes it

more viscous and less likely to penetrate the ACS. This is why HA above 75 mg/mL was not used in this study. Therefore, follow-up studies to determine the threshold of the osteoinductive capacity of HA need to explore other treatments of HA.

Chapter 5. Conclusion

Within the limits of this study, it can be concluded that the bone formation in the 75 mg/mL HA group was superior to the other groups (1, 25, and 50 mg/mL HA and control), thus demonstrating the dose-dependent effect of hyaluronic acid in bone regeneration. However, additional research is required to determine osteoinductive dose thresholds for HA.

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Figure legends

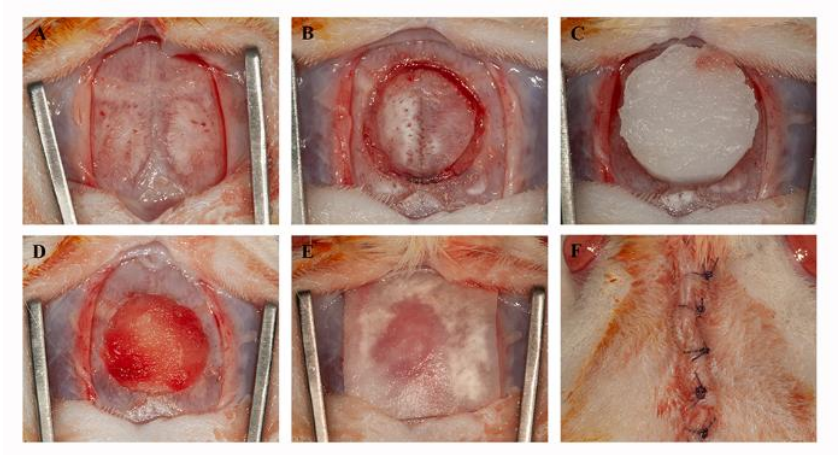

Figure 1. Surgical procedures: (A) A 3-cm midline incision was made through the skin along the sagittal suture of the skull, and the soft tissues and periostea were elevated and reflected. (B) Under saline irrigation, craniotomy defects with an 8-mm diameter were created in rats using a trephine bur. (C) An absorbable collagen sponge (ACS) was cut into the shape of the defect with a diameter of 8 mm, soaked in hyaluronic acid (HA) or saline, and (D) placed in the defect site. (E) A collagen membrane was used to cover the surgical site. (F) The incision was sutured in layers.

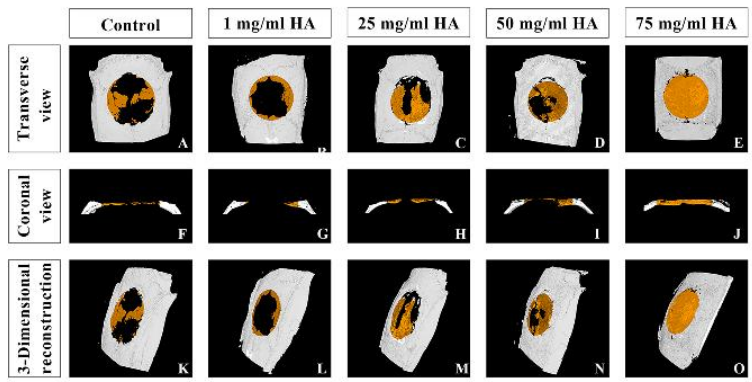

Figure 2. Micro-computed tomography analysis of newly formed bone at the calvarial defects in the control group, 1 mg/mL HA group, 25 mg/mL HA group, 50 mg/mL HA group, and 75 mg/mL HA group at 4 weeks postoperatively. Sectional images with 3-dimensional reconstruction images. (A-E) Transverse view, (F-J) coronal view, and (K-O) 3-dimensional reconstruction. The orange area in the image indicates the volume of interest. Notably, the 75 mg/mL HA group exhibited the most new bone formation among the 5 groups. The control and 1 mg/mL HA groups showed less new bone formation.

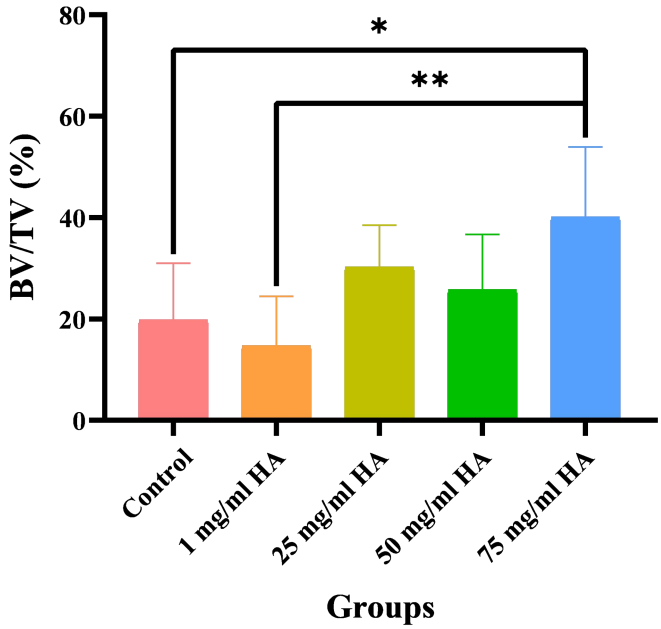

Figure 3. Micro-computed tomography quantitative analysis of newly formed bone at the calvarial defects. The bone volume/total volume ratio (BV/TV) at 4 weeks after defect creation is shown. Notably, the 75 mg/mL HA group induced significantly more bone formation compared with the control (*P<0.05) and 1 mg/mL HA groups (**P<0.01).

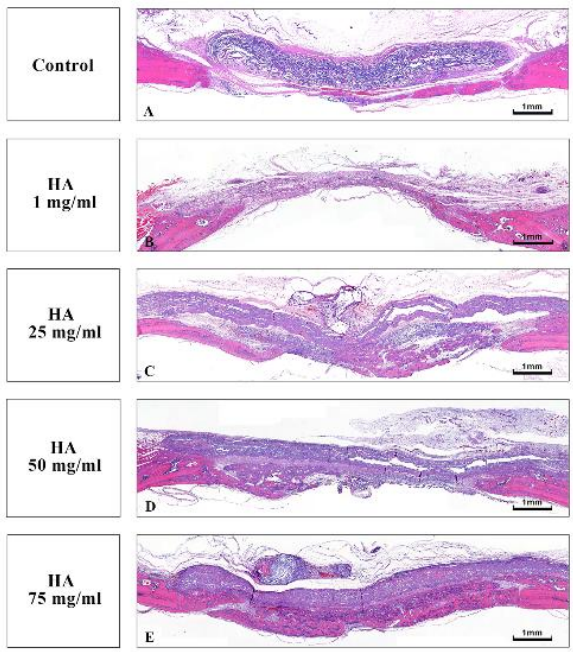

Figure 4. Hematoxylin and eosin-stained histological sections at 4 weeks after defect creation. Representative histological sections show a cross-section of the entire defect with native bone at the edges. (A and B) A small amount of new bone is concentrated in the surgical margins (C and D) Somewhat more new bone was formed. (E) Of all groups, the 75 mg/mL HA group showed the most new bone and blood vessels compared to the other groups.

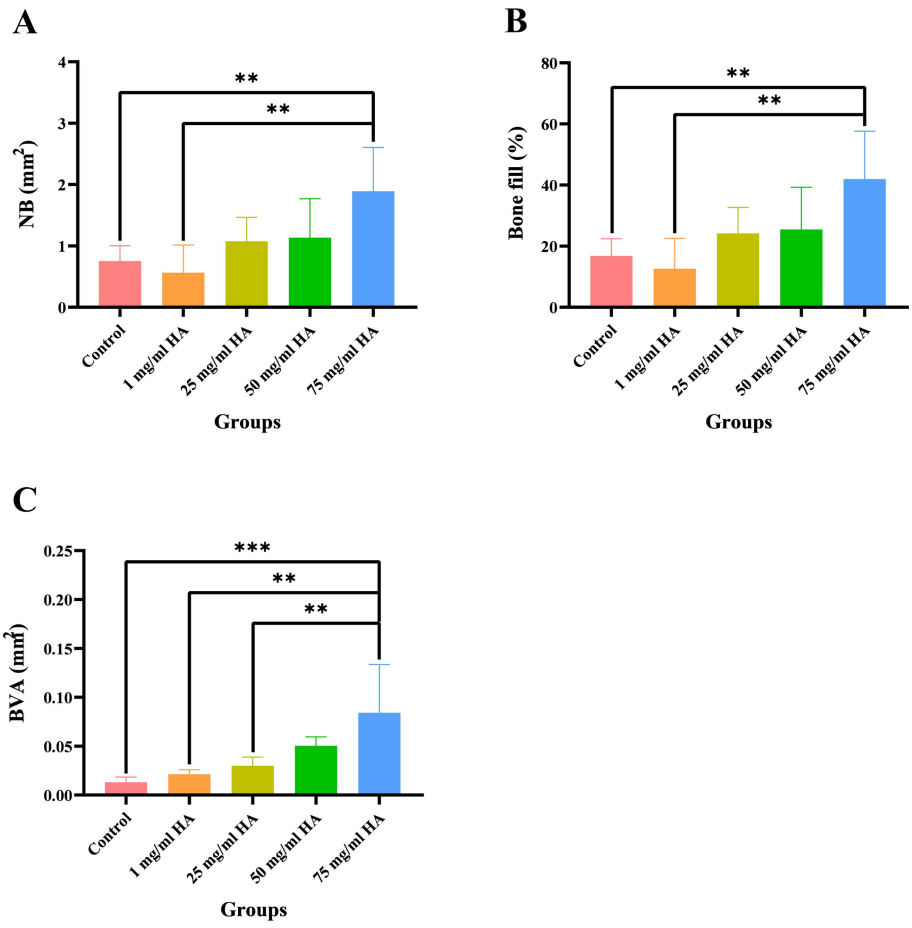

Figure 5. Histomorphometric analysis of new bone formation in the defects. (A) NB, (B) bone fill, and (C) BVA at 4 weeks after defect creation are shown. The NB and bone fill were significantly higher in the 75 mg/mL HA group than in the control and 1 mg/mL HA groups (**P<0.01). The BVA was significantly higher in the 75 mg/mL HA group than control (***P<0.001), 1 mg/mL HA (**P<0.01), and 25mg/mL HA groups (**P<0.01).

NB: new bone area, BVA: blood vessel area

Table legends

Table 1. Histomorphometric results for the 5 study groups at 4 weeks postoperatively

Groups	Control (n=6)	HA (mg/mL)				*P* value
		1 (n=6)	25 (n=6)	50 (n=6)	75 (n=6)	
NB (mm^2)	0.75±0.25	0.56±0.45	1.07±0.39	1.13±0.64	1.89±0.71	0.002
DA (mm^2)	4.49±0.07	4.47±0.05	4.43±0.09	4.39±0.15	4.51±0.10	0.259
Bone fill (%)	16.81±5.60	12.58±9.98	24.19±8.49	25.50±13.80	41.95±15.65	0.002
BVA (mm^2)	0.01±0.01	0.02±0.00	0.03±0.01	0.05±0.01	0.081±0.05	0.000

Data are presented as the mean ± standard deviation. Analysis of variance was performed to determine differences among the 5 groups at a significance level of $\alpha<0.05$.

NB: new bone area, DA: defect area, BVA: blood vessel area

국문초록

랫드 두개골 결손부 골재생을 위한 적정 히알루론산 투여량에 대한 비교 평가

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1. 목 적

본 연구의 목적은 임계크기(Critcal-size)의 랫드 두개골 결함 모델을 사용하여 골 형성에 가장 효과적인 히알루론산(Hyaluronic acid, HA) 용량을 알아보는 것이다.

2. 방 법

서른 마리의 Sprague-Dawley rat를 한 그룹 당 여섯 마리씩 다섯 개 그룹에 무작위로 배정하였다. 랫드의 두개골에 8mm의 임계크기 결함을 형성하였다. 결함 부위에는 서로 다른 투여량의 HA 혹은 생리식염수에 흠뻑 적신 absorbable collagen sponge (ACS) 담체가 삽입되었고, 그 위에 collagen membrane을 덮은 다음 봉합하였다. 본 실험에서는 (1) saline control, (2) 1mg/mL HA, (3) 25mg/mL HA, (4) 50mg/mL HA, (5) 75mg/mL HA와 같은 5개 군이 사용되었다. 모든 동물은 4주 후 희생되었고 각각의 샘플에 대해서는 조직학적, 조직형태학적 분석 및 마이크로컴퓨터 단층촬영이 시행되었다. 조직학적 분석에서는 defect

area (DA), new bone area (NB), bone fill, Blood vessel area (BVA)를 측정하였으며, 마이크로컴퓨터 단층촬영을 통해 얻은 이미지 데이터에서는 bone volume/total volume ratio (BV/TV)를 측정하였다. One-way ANOVA와 Turkey test를 사용하여 그룹 간의 차이를 검증하였다.

3. 결 과

술 후 4주 되는 시점에서 75mg/mL HA 군이 5개 군 중 BV/TV, NB, bone fill 및 BVA가 가장 높게 나타났다. BV/TV, NB, bone fill은 대조군 및 1mg/mL HA 군과 비교 하였을 때 통계적으로 유의한 차이가 관찰되었고 BVA는 대조군, 1mg/mL HA 군 및 25mg/mL HA 군과 비교하였을 때 통계적으로 유의한 차이가 관찰되었다.

조직형태학적 분석 결과 비교적 높은 용량의 HA 군(25mg/mL, 50mg/mL, 75mg/mL)에서 골 형성이 더 활발히 진행되었고, 많은 양의 woven bone을 함유하고 있음을 알 수 있었다. 그리고 75mg/mL HA 군에서 다른 군들보다 상대적으로 더 많은 혈관이 관찰되었다.

4. 결 론

술 후 4주 되는 시점에서 75mg/mL HA 군은 다른 군들(1mg/mL, 25mg/mL, 50mg/mL 및 대조군) 보다 더 많은 양의 골 형성을 보여주었다. 이는 HA가 골재생에 있어 용량 의존성을 갖는 다는 것을 보여준다.

주요어: 골, 골재생, 동물모델, 랫드, 히알루론산

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