2005년도 8월

박사학위논문

Early bone healing of adding Hyaloss™ to grafting materials in osseous defects in rats

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백서의 골 결손부에 이식재와 Hyaloss™를 혼합한 후 초기 골치유

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이 논문을 치의학 박사학위신청 논문으로 제출함.

2005년 8 월 일

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2005년 6월 일

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백서의 골 결손부에 이식재와 Hyaloss^{TM}를 혼합한 후 초기 골치유

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본 연구의 목적은 각종 골 이식재중 치아 회분말과 치과용 연석고를 혼합한 후 골조직의 재생능력을 증가시킬 것으로 여겨지는 여러 제재 중 hyaluronic acid가 주 성분인 Hyaloss^{TM}를 첨가하여 흰쥐의 두개골 결손부에서의 초기 치유과정을 통하여 Hyaloss^{TM}의 역할을 알아보는 데 있다.

실험동물은 동일조건 하에서 일정기간 사육한 체중 200mg 이상의 Sprague-Dawley 흰쥐를 암수 구별없이 사용하였다. 노출시킨 두개골의 정중앙부에 #1/4 round bur를 이용하여 직경 1㎝ 크기의 원형으로 전층골 결손을 야기시킨 후, 제1군은 아무런 이식을 시행하지 않았고, 제2군은 치아회분말과 치과용 연석고를 무게비 2:1로 혼합하여 멸균한 이식재를 멸균 식염수와 혼합하여 이식하였고, 제3군은 치아회분말과 치과용 연석고를 무게비 2:1로 혼합하여 멸균한 이식재를 0.2cc의 Hyaloss^{TM}와 혼합하여 이식하였으며, 제4군은 Hyaloss^{TM}만 이식하였다.

조직 검사를 위해 실험 후 2주와 4주로 나누어 실험군을 희생한 후 매식된 경계부를 포함하여 조직편을 채취한 후, 중성 포르말린 용액에 일정기간 고정하고, 탈회 및 포매과정을 거쳐, Hematoxyline-Eosin으로 이중 염색하여 광학 현미경으로 흡수정도, 신생 골의 형성, 염증반응 유무 등의 치유과정을 분석하였다.

각 주에 따른 군별 비교에서 2주의 경우 전체적으로 유의한(p=0.006) 신생골 형성의 차이를 보였으며, 1-2군간(p=0.009), 1-3군간(p=0.006), 1-4군간(p=0.006) 비교에서 신생골 형성의 유의한 차이가 있었으며, 4주의 경우 역시 전체적으로 유의한(p=0.000) 신생골 형성의 차이를 보였으며, 1-2군간(p=0.004), 1-3군간(p=0.009), 1-4군간(p=0.003), 2-3군간(p=0.010), 2-4군간(p=0.004) 비교에서 신생골 형성의 유의한 차이가 있었다.

임계한계 이상의 골결손을 수복하기 위해서는 골형성 유도물질의 이식이 필요한데, 치아 회분말 단독, Hyaloss^{TM} 단독은 물론 치아 회분말과 Hyaloss^{TM}를 혼합하여 이식할 경우 효과적인 신생골 형성을 기대할 수 있을 것으로 판단된다. 이 중에서도 특히 치아 회분말의 효과가 탁월한데, Hyaloss^{TM}를 섞어서 사용할 경우 신생골 형성 결과가 치아 회분말에는 약간 못 미치지만 비교적 충분한 결과를 기대할 수 있어 치아 회분말의 대용품 또는 혼합물로 사용될 수 있을 것으로 사료된다. 추후 장기간의 투여시 기간별, 농도별 연구가 이에 대한 더 많은 연구가 필요하리라 사료된다.

Introduction

Various biomaterials are used in dental surgery and new biomaterials are continually being developed and introduced. Biomaterials are used for transplantation in oral and maxillofacial surgery, periodontal surgery, and implant surgery; to restore hard and soft tissue defects; for guided tissue regeneration; and for esthetic purposes in plastic surgery.[1-16]

Autografts are the ideal material for reconstructing hard tissue defects. They undergo osteogenesis, osteoinduction, and osteoconduction; do not pose the risk of immune rejection; and require short recovery times. However, only a small amount of tissue can be harvested, and absorption and a secondary defect at the donor site are major drawbacks. To avoid harvesting an autograft and thereby eliminate additional surgical procedures and risks, bone grafting materials and substitutes are used as alternative grafting materials for ridge augmentation.[17] The ideal grafting materials should (1) be biocompatible, non-carcinogenic, and non-allergenic; (2) show early vascularization; (3) be replaced by new host bone tissue; (4) have strength, resist infection, and be sterile; and (5) undergo surface resorption by the host.[11,12]

Tooth ash, which consists mainly of hydroxyapatite, is easily obtained from extracted teeth. Plaster of Paris is easy to obtain and sterilize, is inexpensive, and is absorbed completely. Experimental studies have proved that a mixture of tooth ash and plaster of Paris promotes bone healing while increasing the stability of tooth ash and that the mixture can be used to restore hard tissue defects, for guided bone regeneration, in oral and maxillofacial surgery, and as a barrier membrane.[1-3,6,8-15] The advantages of a mixture of tooth ash and plaster of Paris are (1) the lack of a foreign body reaction, (2) excellent osteoconduction, (3) excellent absorption, (4) possible immediate use, and (5) low cost. The mixture directly binds newly formed bone to increase the stability and firmness of the bone and to prevent granular movement.[1,11,15,16]

The extracellular matrix (ECM) plays an active, complex role in regulating the behavior of the cells that contact it, influencing their development, migration, proliferation, shape, and metabolic functions.[18] The ECM of connective tissue consists of glycosylated proteins connected by glycosidic bonds and includes proteoglycans, mucopolysaccharides, glycosaminoglycans, peptidoglycans, and glycoproteins. They are linked to form groups by hyaluronic acid, which has a strong bonding ability.[19]

Hyaluronic acid is the base material for connective tissues and was discovered in the bovine vitreous. As a polyanionic mucopolysaccharide, it is a biopolymer and is present in bone cells as a component of the ECM.[20-22] Hyaluronate, which is widely distributed in connective tissues, was chosen as the model glycosaminoglycan for these studies and was bound to insoluble type I collagen fibers of polydispersed size.[23]

Hyaluronic acid is important in many biological processes, such as tissue hydration, proteoglycan organization in the extracellular matrix, cell differentiation, cell behavior, and tissue repair. In recent years, hyaluronic acid has been used for clinical purposes in many different pathological conditions, including eye surgery, osteo-arthritis, wound repair, and cosmetic surgery.[24] Techniques are available for obtaining large quantities of pure hyaluronic acid from natural sources. In addition, hyaluronic acid can be modified chemically and physically and can be fabricated into a number of physical forms, including fibers, strands, ropes, films, woven and non-woven material, sheets, and sponges.[25]

This study examined the role of Hyaluronic acid during early bone formation in rat skull defects.

Materials and Methods

Study Animal

This study was approved by the Animal Research Committee of Chosun University. Twelve-week-old Sprague-Dawley rats were selected for the study.

Eight-mm-diameter calvarial critical-size defects were created in rats. A critical-size defect is defined as the smallest intraosseous wound in a particular bone and species of animal that will not heal during the lifetime of the animal.

Forty-eight rats were randomly assigned to four groups, and each group was further divided into two subgroups: 2 weeks and 4 weeks after implantation. The defect was filled in different manners: Group 1, non-graft group; Group 2, tooth ash-plaster graft group; Group 3, Hyaloss™ and tooth ash-plaster graft group; and Group 4, Hyaloss™ graft group. Histologic sections were obtained for histomorphometric analysis of the defects at 2 and 4 weeks after surgery.

Study Materials

Tooth ash was prepared from healthy teeth extracted from humans by washing the teeth in saline solution, ashing them in a furnace at 1200° C, and grinding the product into a powder using 100 mesh (0.149 mm). The high temperature eliminates any virus, bacteria, and fungus. The resulting tooth ash was mixed with plaster of Paris (calcium sulfate hemihydrate, Gypsum Co.,USA) in a weight ratio of 2:1. All materials were sterilized with ethylene oxide before implantation, and physiological saline solution was used to mix the implants.

Hyaloss™ Matrix

Hyaloss™ matrix comes in a package complete with an insert and a heat-sealed PET blister pack with an aluminum backing. Each blister can be opened singly and contains a strip of sterile fibers, about 1.5 cm long, entirely composed of HYAFF®.. The product is sterilized by radiation. The contents of each blister are to be mixed with 0.5cc of bone sample. It is intended for use as an adjuvant in the surgical application of bone grafts for the treatment of dental defects. On contact with blood or sterile saline, the fibers turn into a gel that

facilitates the application of bone fragments. The gel is biodegradable and is absorbed within about one week, enabling the graft to take naturally and allowing bone regeneration. The biodegradable, biocompatible gel is mixed with bone fragments to form a paste that can easily be molded to fit the bone defect. The dentist can regulate the quantity of sterile saline or blood to give the gel the required consistency.

Implantation

Each rat was anaesthetized using ether inhalation. The head was shaved and sterilized using the conventional method, and 2% lidocaine HCl containing 1:100,000 epinephrine was injected for hemostatic purposes. An incision was made along the midline of the head to expose the skull. An 8-mm-diameter hole was drilled in the skull, removing the entire layer of the skull, by using a 1/4 round bur. An already prepared mixture of grafting materials were used to close the defect. The skin was then sutured over the skull. An intramuscular injection of 0.05 ml/kg gentamicin (Samwoo Pharmaceuticals) was administered to prevent infection after surgery. The rats were sacrificed at 2 and 4 weeks after surgery.

Histomorphometric Analysis

After a rat was sacrificed using excess ether inhalation, a bone sample was obtained from around the implant site, fixed in 10% neutral formalin for 72 h, and decalcified in nitric acid for 4 h. The bone sample was cut into 3-mm-thick sections, which were washed in running water. Each bone sample was treated using an autoprocessing machine (Hypercenter XP, Shandon, UK). After paraffin embedding, each section was cut into 4- to 5-μm slices, which were stained with hematoxylin-eosin and Goldner's trichrome and were observed under an optical microscope.

Computer-assisted histomorphometry was used to measure the amount of bone formed at the defect site. Images were taken using a Polaroid digital microscope camera (Polaroid, Cambridge, MA, USA) and were analyzed using Image Pro Plus (Media Cybernetics, LP, Silver Spring, MD). Images of each tissue sample were analyzed.

Quantitative Analysis

The Kruskal-Wallis test was used to compare the subgroups and groups overall; the Mann-Whitney test was used to compare the two subgroups within each group; and the Wilcoxon signed-ranks test was used to compare the groups at each time period. Values of $p < 0.05$ were considered statistically significant.

Results

Group 1

Group 1 at 2 weeks (Fig *1*)

New bone formation was slight and was restricted to a limited area at the edge. The rest of the area of the bone defect was filled with fibrotic tissue.

Group 1 at 4 weeks (Fig *2*)

No significant difference was seen compared with the results at 2 weeks. New bone formation was observed around the edge of the bone defect.

Group 2

Group 2 at 2 weeks (Fig *3*)

Anastomosing trabeculae were obvious and occurred in a centripetal growth shape with abundant growth at the edge of the bone defect and thinner and scarcer growth toward the center of the bone defect, as if new bone formation had started from the edge and grew into the center. By contrast, implanted chips were found in the top and bottom portions of the area of new bone formation or fibrotic tissue with poor new bone formation.

Group 2 at 4 weeks (Fig *4*)

Abundant new bone formation was observed compared with the growth at 2 weeks, and the difference was statistically significant.

Group 3

Group 3 at 2 weeks (Fig *5*)

Anastomosing trabeculae showed centripetal growth, and some portions exhibited implanted chips and fibrotic changes without new bone formation.

Group 3 at 4 weeks (Fig *6*)

Although new bone formation was excellent compared with that at 2 weeks, the overall bone growth was weak and did not differ significantly from that at 2 weeks.

Group 4

Group 4 at 2 weeks (Fig *7*)

Anastomosing trabeculae showed centripetal growth, and no fibrotic changes were seen in some portions.

Group 4 at 4 weeks (Fig *8*)

New bone formation was better than that at 2 weeks, but the difference was not significant. In some cases, new bone formation was excellent.

The new bone formation activities at 2 and 4 weeks are summarized in Table 1.

Table 1. RESULTS FOR NEW BONE FORMATION (unit: ㎟)

	Group 1	Group 2	Group 3	Group 4
2 wks	0.038 ± 0.025	1.130 ± 0.441*	1.29 ± 0.848*	0.700 ± 0.585*
4 wks	0.067 ± 0.035	2.283 ± 0.256**	1.15 ± 0.252*	1.000 ± 0.253**

Group 1, non-graft group; Group 2, tooth ash-plaster graft group; Group 3, Hyaloss™ and tooth ash-plaster graft group; Group 4, Hyaloss™ graft group.

*Statistically more significant new bone formation, $p < 0.05$.

**Statistically more significant new bone formation in the ash group than the control group, $p < 0.005$.

Comparisons among the groups at the same time period revealed that the amount of overall bone formation was significantly different among the groups at both 2 and 4 weeks. At 2 weeks, there were significant differences between Groups 1 and 2 (p=0.009), Groups 1 and 3 (p=0.006), and Groups 1 and 4 (p=0.006). At 4 weeks, there were significant differences in overall bone formation between Groups 1 and 2 (p=0.004), Groups 1 and 3 (p=0.009), Groups 1 and 4 (p=0.003), Groups 2 and 3 (p=0.010), and Groups 2 and 4 (p=0.004).

FIGURE 1. Photomicrograph of Group 1 at 2 weeks. Tiny areas of new bone formation are seen around the defect margin (arrows) (hematoxylin and eosin stain, original magnification X40).

FIGURE 2. Photomicrograph of Group 1 at 4 weeks. Tiny areas of new-bone formation are seen around the defect margin (arrows) (hematoxylin and eosin stain, original magnification X40).

FIGURE 3. Photomicrograph of Group 2 at 2 weeks. Centripetal, anastomosing, woven bone formation is present. Implanted chips (arrows) are also seen in the lower portion (hematoxylin and eosin stain, original magnification X40).

FIGURE 4. Photomicrograph of Group 2 at 4 weeks. Marked centripetal, woven bone formation is seen. Pre-existing bone (asterisks) is present (hematoxylin and eosin stain, original magnification X40).

FIGURE 5. Photomicrograph of Group 3 at 2 weeks. Centripetal, anastomosing, woven bone formation is seen. Implanted chips (arrows) and fibrosis without new bone formation are identified on the right side (hematoxylin and eosin stain, original magnification X40).

FIGURE 6. Photomicrograph of Group 3 at 4 weeks. Centripetal, woven bone formation is present. Implanted chips (arrows) without new bone formation are seen on the right side (hematoxylin and eosin stain, original magnification X40).

FIGURE 7. Photomicrograph of Group 4 at 2 weeks. Centripetal, woven bone formation is identified. Pre-existing bone (asterisk) is present (hematoxylin

and eosin stain, original magnification X40).

FIGURE 8. Photomicrograph of Group 4 at 4 weeks. Centripetal, anastomosing, woven bone formation is greater than that at 4 weeks in Group 1. Pre-existing bone (asterisk) is seen (hematoxylin and eosin stain, original magnification X40).

Discussion

Hyaluronic acid (HA) is a macromolecule consisting of N-acetyl-β-D-glucosamine and β-D-glucuronic acid residues linked at the 1,3 and 1,4 positions, respectively.[26,27] It is synthesized in the plasma membrane of fibroblasts and other cells by adding sugars to the reducing end of the polymer, while the non-reducing end protrudes into the pericellular space. The polysaccharide is catabolized locally or is carried by the lymph to lymph nodes or the general circulation, from which it is cleared by the endothelial cells of the liver sinusoids.[18,27]

Hyaluronic acid is one of the most highly conserved molecules over the course of evolution. Regenerating tissues are rich in hyaluronic acid, which contributes to a large variety of cell functions essential for tissue repair, such as the inflammatory response, cell migration, cell proliferation, and the organization of extracellular matrix. Hyaluronic acid also plays a major role in angiogenic processes[22] and serves multiple purposes in mediating the processes that lead to tissue repair. One of its characteristic features is that its biological functions can be attributed to both its physicochemical characteristics and specific interactions with cells and the extracellular matrix.[28,29]

Hyaluronic acid is an extremely hydrophilic, linear polymer and is a viscous gel in its natural form. Consequently, it lacks the structural characteristics that would make it suitable for use in medical-surgical devices. For treating slow-healing wounds, high concentrations of hyaluronan can be used; in hyaluronan, the chemical characteristics of hyaluronic acid are modified, while maintaining all of its natural biological properties. Using this conservative chemical modification, hyaluronic acid can be transformed into a biopolymer called HYAFF® .[22]

HYAFF® is an ester of hyaluronic acid, obtained by esterifying the carboxyl group of glucuronic acid with benzyl alcohol. The esterification process reduces the hydrophilic properties of the molecule and transforms hyaluronic acid into a molecule that is less water soluble. By altering the degree of solubility, it is possible to produce medical devices made of pure hyaluronic

acid in a solid form, such as Hyaloss™ matrix, which is made of HYAFF® fibers.[22]

HYAFF® matrix is a completely bio-absorbable biopolymer that can be used as an adjuvant in dental surgery involving bone grafts to correct dental defects. It first acts as a gelling agent, facilitating the application of bone tissue fragments, and then exploits the properties of hyaluronic acid to facilitate repair of the defect. The gel forms at room temperature and can be molded to fit the defect, making it fully flexible for use.

The topical application of hyaluronic acid accelerates wound healing with reduced tissue fibrosis. This may be related to the fact that embryonic skin, which has a high hyaluronic acid content, exhibits little scarring when wounded.[29]

With regard to hyaluronic acid and the bone formation process, in 1914, Pfaundler identified "Kalksalzfanger (lime salt catcher)," an unknown compound containing abundant calcium, in the bone formation process and suggested that calcium bonding is important during bone formation.[30] In the 1960s, researchers believed that GAGs and proteoglycans were biologically related to the bone formation process. In a cytological study, Cuervo et al reported in 1973 that sulfide proteoglycan was condensed to prevent bone formation.[31,32] In 1976, Howell and Pita reported that the concentrations and sizes of proteoglycans are closely related to cartilage or bone formation. In 1985, Chen and Boskey et al emphasized that proteoglycans and the calcification process are closely related and reported that they limit the formation of hydroxyapatite in cytologic and animal studies.[33,34] Boskey and Dick reported that hyaluronic acid and hydroxyapatite do not interactively prevent crystal proliferation and that the non-sulfide proteoglycan, hyaluronic acid, expeditiously provides a mineralization environment, suggesting that sulfate controls mineral formation.

After creating an artificial defect in rat skulls, implanting collagen/hyaluronate matrix, and observing the defect site 4 weeks later, Liu et al[35] reported the formation of a hematopoietic marrow cavity, with reversal lines and a bone regeneration scaffold at the bone defect site. Cho et al[36] reported that hyaluronic acid showed active bone formation compared with

controls, especially at a concentration of 1.0 mg/ml, indicating the direct ability of this chemical for bone formation during bone cell differentiation. As hyaluronic acid promotes the healing of various tissues, it should also promote the regeneration of bone tissue, and it is expected that hyaluronic acid can be developed as an accelerator for regenerating bone tissue.

In this study, we implanted a sterilized grafting material composed of tooth ash and plaster of Paris mixed in a 2:1 ratio by weight, after adding sterile saline solution, in Group 2. In this group, significant anastomosing trabeculae formed thickly and densely at the edge of the bone defect, but became scarcer toward the center of the defect. Conversely, implanted chips were found above and below newly formed bone, and fibrotic tissues showed little new bone formation. In order to restore bone defects exceeding the critical limit, it is necessary to implant a graft material to guide bone formation. This might consist of tooth ash alone, Hyaloss™ alone, or a mixture of tooth ash and Hyaloss™. Of these materials, tooth ash was the best. When Hyaloss™ was mixed with tooth ash, new bone formation was satisfactory, although slightly less than that with tooth ash. Therefore, Hyaloss™ can replace or be mixed with tooth ash. Further studies are needed on the effects of its long-term use and optimal concentrations.

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