CELLAPURE

EFFECT OF HUMAN AMNIOTIC FLUID ON BONE HEALING

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Effect of Human Amniotic Fluid on Bone Healing

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Background. Bone healing continues to pose challenges for researchers and clinicians working in the field of plastic surgery. Complete bone regeneration cannot be obtained in critical size osseous defects without the application of osteogenic or osteoinductive bone material. In this study, we hypothesized that because extracellular matrix components are known to play a major role in the first steps of healing during bone or injury healing and because hyaluronic acid as chondroitin sulfate is recognized as an osteogenic compound without osteoinductive activity, human amniotic fluid, which contains high concentrations of hyaluronic acid, hyaluronic acid-stimulating activator, and other factors, might accelerate bone healing when applied subperiosteally to rabbit calvarial defects.

Materials and methods. We created 20 calvarial defects in 10 12-week-old New Zealand white rabbits who were divided into 2 groups. Group 1 defects were instilled with human amniotic fluid, whereas the group with contralateral defects, i.e., group 2, were given with same amount of normal saline solution. We then measured the density of the bone that formed over the defects using computed tomography at the third, fourth, fifth, and sixth weeks postoperatively. After this period, the defects were harvested for histopathologic evaluation.

Results. The defects from group 1, which were treated with human amniotic fluid, showed significantly higher ossification than the group 2 defects, which were instilled with saline solution. Histological examination at 6 weeks postoperatively revealed that the defects treated with human amniotic fluid (group 1) had superior ossification compared with the control group defects (group 2).

Conclusion. Because of its positive effects on bone healing and also because of its ability to be stored in deep freeze if made cell-free, human amniotic fluid would appear to be a useful adjunct in the treatment of bone healing. © 2005 Elsevier Inc. All rights reserved.

Key Words: human amniotic fluid; bone healing; hyaluronic acid.

INTRODUCTION

Oncologic surgery, trauma, and congenital disorders often leave patients with large bony defects. Complete bone regeneration cannot be obtained in critical-sized osseous defects in the absence of an application of osteogenic or osteoinductive bone material. The best experimental results for most bone deficiencies are provided by osteoinductive molecules, such as bone morphogenetic proteins [1], which are powerful enhancers of bone healing. Other molecules may be used to heal bone defects: TGF-β1, heparan sulfates, carboxymethyl-benzylamide-sulfonated dextrans, heparin-binding growth factors, or heparin sulfate proteoglycans are major components of the extracellular matrix.

Hyaluronic acid (HA) is a linear polysaccharide with a high molecular weight. It is found in all extracellular matrices and has the same structure in all species. It has a unique influence on cell differentiation, motility, and cell adherence and has been suggested to have anti-inflammatory effects [2]. If HA is administered during surgery, scar formation is prevented. It influences and enhances tissue regeneration through its ability to retain large amounts of water [3, 4]. Locally applied HA does not result in any phagocytosis or immunological reactions. HA has been reported to increase osteoblastic bone formation in vitro through increased mesenchymal cell differentiation and migration.

Human amniotic fluid (HAF), obtained by amniocentesis during the second trimester of gestation, contains high molecular weight HA in high concentrations and HA-stimulating activator (HASA) [5, 6]. It has been showed that HASA, which is present in HAF, stimulates the wound to increase the production of endogenous HA.
[7, 8]. Thus, HAF may increase both endogenous and exogenous HA in the application region. HA is known to reduce scar formation by inhibiting lymphocyte migration, proliferation and chemotaxis, granulocyte phagocytosis and degranulation, and macrophage motility [9]. HAF has been reported to prevent peritendinous adhesion formation and enhance new cartilage formation. The present study was designed to investigate the efficacy of HAF as a source of HA to enhance new bone formation and healing.

MATERIALS AND METHODS

Ten New Zealand male rabbits with a mean age of 12 weeks and weighing 1500-1700 g were used in this study. All procedures were performed in the Experimental Animals Breeding and Research Centre of the Faculty of Medicine of Karadeniz Technical University. Animal care was conducted with the approval of the Animal Experimental Ethics Committee of Karadeniz Technical University, Trabzon, Turkey.

All rabbits were anesthetized with an intramuscular (IM) injection of ketamine hydrochloride (Ketalar®, Pfizer, Istanbul, Turkey) 35 mg/kg body weight and xylazine hydrochloride (Rompun®, Bayer Healthcare, Leverkusen, Germany) 5 mg/kg body weight. A single intramuscular injection of Cefuroxime (GlaxoSmithKline, Research Triangle Park, NC), 30 mg/kg body weight, was given before surgery for prophylaxis. The rabbits were placed in the prone position and the skin overlying the calvaria was shaved and swabbed before and after surgery with a 10% povidone iodine solution (Batticon®, Adeka, Samsun, Turkey). The calvariae were exposed through bilaterally paramedian skin incisions. By bilaterally created pockets that were totally isolated from each other, periosteal flaps were elevated, and 5-mm-diameter defects were made in the cranial bones by means of a standardized trephine cutting bur and drill. The periosteal flaps were carefully positioned in place using resorbable suture material (Vicryl, Johnson &Johnson Intl, Brussels, Belgium).

The defects were assigned to 2 groups. Before closing the skin incisions, 0.3 mL of HAF was instilled into the cranial defect underneath the periosteal flap located on the right side of the cranium (group 1, treatment group), and a normal saline solution (0.3 mL) was introduced as the same manner into the subperiosteal space located on the other side, which acted as a control (Group 2). The skin incisions were closed with interrupted sutures using nonabsorbable suture (Trofilen, Doğsan, Trabzon, Turkey). HAL was donated from Samsun, Turkey). The calvariae were exposed through bilaterally paramedian skin incisions. By bilaterally created pockets that were totally isolated from each other, periosteal flaps were elevated, and 5-mm-diameter defects were made in the cranial bones by means of a standardized trephine cutting bur and drill. The periosteal flaps were carefully positioned in place using resorbable suture material (Vicryl, Johnson &Johnson Intl, Brussels, Belgium).

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The density of the bone formed over the defect was measured by serial 3D computed tomography (CT) scans, obtained from all rabbits at 3, 4, 5, and 6 weeks postoperatively. All rabbits were tranquilized with an IM injection (10 mg/kg body weight) of ketamine. The scans were taken in the coronal plane by means of a multidetector CT scanner (Somatom, Volume Zoom, Siemens, Forchheim, Germany). Scan parameters were: DFOV = 8.2–8.8 cm; mA = 120-150; kV = 120 at a thickness of 0.5 mm. After scanning, multidetector CT data sets were transferred to an independent workstation (Virtuso, Siemens, Germany) for postprocessing. All measurements were taken by one individual who was blinded as to group identity.

Histological Analysis

All rabbits were killed at 6 weeks after surgery with an IV overdose (45 mg/kg) of pentobarbital. After harvesting and decalcification of the defects, the specimens were embedded in paraffin. Serial 5-mm-thickness sections were cut from the center of the defect and prepared for histological staining with hematoxylin and eosin. All of the slides were analyzed by the same person, who was blinded as to which treatment solution was used.

Statistical Analysis

Data analysis was performed using the statistical software package SPSS (version 10.0 for Windows; SPSS, Inc., Chicago, IL). The mean density of bone that formed over the defects were evaluated using the Student’s t test. Statistical significance was indicated at $P < 0.05$.

RESULTS

All rabbits tolerated the surgical procedures well. No significant reductions in body weights were noted, and no postoperative infections were observed. Skin tissues were in fair condition, and scars were not proliferative.

Gross Findings

After 6 weeks, minimal-to-no adhesion or fibrosis was observed on the right-sided defects (Group 1, treatment group) whereas the contralateral defects (Group 2, control group) showed evident periosteal reaction. Under gross inspection, defects in the treatment group were smaller and more amorphous than the control group defects.

Radiological Evaluation

The scans were revealed that evidence of ossification was substantially increased in treatment group compared with the control group (Figs. 1-4). The mean density of the new bone formed on the treatment group defects through the third to sixth weeks was $104 \pm 12$, $148 \pm 14$, $180 \pm 13$, and $220 \pm 18$ Hounsfield Units (HU) respectively, whereas the density of the new bone formed on the contralateral control side defects was $84 \pm 13$, 101
± 17, 129 ± 16, and 140 ± 18 HU, respectively. Multiple-comparison tests revealed that the differences in bone density between groups were statistically significant during all time intervals \( (P < 0.001; \text{Fig. 5}) \).

Histopathologic Evaluation

Histological examination at 6 weeks after surgery revealed that the defects treated with HAF had the greatest ossification compared with the control group defects (Figs. 6 and 7). Control defects showed extensive fibrous tissue ingrowth and poor ossification, mainly among the margins. In contrast, defects treated with HAF showed bicortically new bone formation extending from the lateral margins.

DISCUSSION

Bone healing continues to pose challenges for researchers and clinicians working in the field of plastic surgery. Although researchers have developed numerous experimental methods by which to improve bone healing, the repair of large bony defects requires the surgical transfer of bone from donor to wound site, which is considered to be the clinical “gold standard.” Alternative treatments used in the healing of bone defects, including fracture, nonunion, fusion, implants, and congenital pseudoarthrosis, may benefit from the use of biological methods, such as osteoconductive materials and osteoinductive growth factors and substances [10, 11]. Biological agents incorporated in fracture management, fusion, and bone grafting include bone morphogenetic proteins [1], which are powerful enhancers of bone healing, TGF-b1, heparan sulfates, carboxymethyl-benzylamide-sulfonated dextrans, heparin-binding growth factors, or heparan sulfate proteoglycans, which are a major component of the extracellular matrix.

In this study, we hypothesized that because extracellular matrix components are known to play a major role in the first healing steps [12, 13] and during bone or injury healing [14] and because HA as chondroitin

![FIG 2. Three-dimensional image of the same defect seen on Fig. 1. (Color version of figure is available online.)](image)

![FIG 3. Density of the new formed bone 6 weeks postoperatively in group 2 (control group). (Color version of figure is available online.)](image)

![FIG 4. Three-dimensional image of the same defect seen on Fig. 3. (Color version of figure is available online.)](image)

![FIG 5. Group 1 defects, which were treated with HAF, showed significantly higher ossification than the group 2 defects. (Color version of figure is available online.)](image)
sulfate is recognized as osteogenic compound without osteoinductive activity [15, 16], HAF, which contains high concentrations of HA, HASA, and other factors [9, 17, 18] might accelerate bone healing when applied subperiosteally in to the rabbit calvarial defects.

The observed healing in the rabbit cranium, because of the structure of the cranial bone within rabbits, is a strong indicator of clinical relevance. With a bone marrow space sandwiched between two cranial plates, the rabbit cranium has a structure similar to all higher animal species. Therefore, the strong healing response is suggestive of the healing potential in larger species. The rabbits own amniotic fluid was not used in this experimental study because similar amounts of amniotic fluid (0.3 mL for each rabbit) would be difficult to obtain from pregnant rabbits. Furthermore, the variations in the concentrations of HA, HASA, and other factors in HAF in women during pregnancy are already known based on data from previous studies [5–8]. As we hypothesized previously, the mean density of the newly formed bone over the calvarial defects in group 1, which was treated with HAF, was significantly higher when compared with group 2, the control.

The beneficial effect of HA and HASA containing HAF on new bone formation in bone wound healing can be related to the fact that HA may play an important role in the binding of growth factors and their protection by reducing enzymatic degradation [19, 20] and in the formation of a large and physically stable coagulum. HA has been proven to have a positive effect on differentiation, migration, and invasion of various cell types because it proves a suitable substratum for cell migration in relation to its physicochemical (negative charges) properties [15, 21, 22].

HA may have another beneficial effect on new bone formation in bone wound healing. It is well known that various locally released growth factors have stimulatory effects on tissue repair. For instance, epidermal growth factor and transforming growth factor-beta (TGF-b) strongly stimulate collagen production and fibroblast proliferation in vivo [23, 24]. In particular, TGF-b strongly increases bone formation in vivo and in vitro by stimulating the proliferation and differentiation of osteogenic cells [25, 26]. It also was reported that the partially purified factor obtained from regenerating bone marrow stimulated fetal long-bone elongation and DNA synthesis in osteoblastic calvarial cells in vitro [27]. HAF is known to be rich in these growth factors [5–8]. HA may have a role in maintaining these factors within the local environment. Taken together, these data suggest that high-molecular HA functions by effectively retaining osteoinductive growth factors within the local environment by virtue of its physicochemical properties; it is capable of accelerating new bone formation during bone wound healing through stimulation of osteogenic cell differentiation.

HA and HASA-containing HAF forms an attractive extracellular matrix for the direct adhesion of osteoblasts, osteoprogenitor cells, and pericytes and may protect the growth and hormonal factors associated with the healing process. The high binding capacity of calcium by HA could also be another important parameter in its efficiency to induce higher bone healing [28].

Human amniotic fluid contains high concentrations of high molecular weight HA during the second trimester of gestation [5, 6, 18]. The presence of HA provides for a healing process through regeneration and growth rather than scarring and fibrosis. In recent studies, it was reported that HA at a concentration of 19 mg/mL and a molecular weight of $6 \times 10^6$ significantly limited adhesion formation [29]. The concentration of HA in HAF
decreases during gestation, measuring approximately 20 mg/L between weeks 16 and 20 and 1 mg/L at week 30 [5, 6]. Therefore, we preferred to obtain HAF during the second trimester. In addition to HA, HAF contains HASA, which stimulates the wound to increase production of endogenous HA [17]. Therefore, HAF would increase both endogenous and exogenous HA in its region of application. The presence of both HA and HASA in HAF would explain the higher density of new formed bones in group 1 treated with HAF. However, the growth factors contained in HAF may explain the observed acceleration of the bone formation in the experimental group.

In our study, the HAF was centrifuged in high speeds and used cell-free; thus, we think that this valuable fluid may be stored in deep freeze in this manner. HAF obtained from the donors who are investigated for HbsAg, HBV- and HIV-antibodies may even be commercially marketable and used as allograft without any risk of reaction.

REFERENCES


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