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The Effects of Human Amniotic Fluid and Different Bone Grafts on Vertebral Fusion in an Experimental Rat Model

Mithat Oner, MD[1], Turan Cihan Dulgeroglu, MD[2], Ibrahim Karaman, MD[2,*], Ahmet Guney, MD[1], Ibrahim Halil Kafadar, MD[1], Sevki Erdem, MD[3]

[1] Department of Orthopaedics and Traumatology, Erciyes University Medical Faculty, Kayseri, Turkey
[2] Department of Orthopaedics and Traumatology, Dumlupınar University Medical Faculty, Kütahya, Turkey
[3] Department of Orthopaedics and Traumatology, Medeniyet University Medical Faculty, Istanbul, Turkey

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ABSTRACT

Objective: The high risk of nonunion represents a challenge in vertebral surgery, thus stimulating new strategies to improve fusion rates. We investigated the effect of 2 different bone grafts and amniotic fluid application on radiologically and histologically evaluated vertebral fusion in an experimental rat model.
Materials and methods: Forty-eight 24-week-old Sprague Dawley rats were included and assigned into 1 of 4 groups: allograft group, allograft plus human amniotic fluid group, demineralized bone matrix (DBM) group, or DBM plus human amniotic fluid group. After decortication and L4–L6 spinal fusion, study treatments were applied. Fusion in each rat was examined radiologically and histologically 8 weeks after the intervention.
Results: The group that received only allograft had better radiologic scores (median = 3.5; range = 3–4) when compared with the group that received only DBM (median = 2; range = 1–4) ($P = 0.002$); however, histologic scores did not differ. When amniotic fluid was added to the grafting, allograft-based treatments performed better than DBM-based treatments both on radiologic (median = 4; range = 3–4 vs median = 3; range = 3–4; $P = 0.003$) and histologic (median = 7; range = 6–7 vs median = 5; range = 3–6; $P < 0.001$) evaluation. Addition of amniotic fluid did not result in better outcomes in the rats that received DBM-based treatments but based on histologic evaluation, rats that received allograft-based treatments benefited from this application.
Conclusions: Amniotic fluid seems to have an enhancing effect on posterior spinal fusion, particularly when combined with allograft.

Introduction

Advances in the field of vertebral surgery have inevitably placed more significance on fusion surgery, which is still associated with a 10% to 15% risk of nonunion even in the presence of internal fixation.[1] In addition, it is a well-known fact that nonunion often requires revision surgery due to patient dissatisfaction.

Vertebral fusion demands a concerted effect of certain biologic and mechanical factors.[1] Although the former of these includes the extraction of joint cartilage, decortication, grafting, and immobilization of the segment to be fused, the latter involves the use of fixation equipment such as rods, plaques, wires, hooks, plasters, corsets, and a variety of apparatuses.[2] Reinforcing the fusion with solid internal fixation does not exclude the possibility of nonunion, again placing increasing emphasis on biologic factors.

A number of studies have been undertaken to investigate alternative strategies, particularly looking at alternatives to bone grafts at a clinical level,[2] due to the high rate of nonunion and donor site morbidity following the use of autografts in primary spinal fusion surgery.[2] In this regard, demineralized bone matrix (DBM) represents a readily available graft alternative with osteoinductive potential that has shown promising results in several studies.[3,4]

Several mediator molecules with anabolic effects such as the transforming growth factor, fibroblast growth factor (FGF), platelet-derived growth factor, interleukin-1, and interleukin-6 can also provide additional benefit in these procedures.[5] Potentially similar growth and trophic factors include insulin-like growth factor (IGF)-I and IGF-II and epidermal growth factor. Specifically, IGF-I and IGF-II are growth factors that are known to be associated with matrix synthesis during the bone recovery phase.[6]

Address correspondence to: Ibrahim Karaman, MD, Department of Orthopaedics and Traumatology, Erciyes University Medical Faculty, TR-38039, Kayseri, Turkey.
E-mail address: drikaraman@gmail.com (I. Karaman).

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In addition, amniotic fluid has been reported to be a rich source of certain extracellular macromolecules such as epidermal growth factor, IGF-I, IGF-II, FGF, fibronectin and laminin,[7,8] hyaluronic acid (HA) (which is a high molecular weight polysaccharide that is abundant in body fluids and in connective tissue), chondroitin sulphate, and an HA activating factor.[7,8] HA is particularly found in soft connective tissues, with some osteoblastic bone-forming effect.[7] The role of amniotic fluid, which has a variety of biologic features in vertebral fusion, has been the subject of very few studies until now.

We investigated the effect of bone grafts (allograft or DBM) and amniotic fluid on vertebral fusion in an experimental rat vertebral fusion model.

Materials and Methods

Experimental animals and study groups

The study conformed to the Turkish national recommendations of the ethics committees for animal research, in line with the European Commission Directive 86/609/EEC for animal experiments. A total of 48 Sprague Dawley rats with a mean weight of 250 g (range = 200–300 g) and age of 24 weeks were included in this study. Animals were placed in cages of 2 and were kept at a stable temperature of 20°C to 24°C with 12 hours of dark and 12 hours of light cycles. Rats were assigned into 4 groups consisting of 12 rats in each: Group I had allograft only, Group II had allograft plus human amniotic fluid, Group III had DBM only, and Group IV had DBM plus human amniotic fluid application. Fusion in each rat was examined radiologically and histologically 8 weeks after the experimental application of the study treatments following experimental decortication and spinal fusion between the fourth and sixth vertebrae.

Preparation of allografts and DBM

To obtain allografts and DBM, 8 rats that were not included in the study were killed, after which both iliac wings, femur, and tibia were stripped off from the soft tissues. Iliac wings were used to obtain allografts.

To prepare DBM, femurs and tibias were frozen at −70°C after removal of the soft tissues. Sterilization was carried out by ethylene oxide. Fragments of 0.5 mm were dissected to obtain DBM. They then were ground to achieve fragments with an average dimension of 106 to 500 μm. The decalcification process was completed by storing the material for 16 hours at 4°C in 0.6 normal hydrochloric acid (N HCL) (100 g/2 L). Materials were then washed in sterile water and soaked in 70% ethanol. DBM was dried using a vacuum dryer overnight, sterilized with ethylene oxide, and kept at −70°C.

Preparation of amniotic fluid

Amniotic fluid was obtained from pregnant women attending the obstetrics outpatient unit in our hospital who completed 20 weeks of pregnancy and signed an informed consent. Centrifugation was performed using a Heraeus Sepatech Megafuge 1.0R (Langenselbold, Germany) device at 4300 revolutions/min for 15 minutes. About 0.1 cc precipitate was obtained from this procedure. The remaining 8 cc supernatant was taken and kept at −20°C. Amniotic fluid to be used in surgery was thawed by keeping at room temperature for 20 minutes.

Surgical methods and follow-up

In anesthetized rats in the prone position, a surgical midline incision was made on the lumbar region along the spinous processes. After skin, subcutaneous tissue, and fascia were incised, the longissimus lumbarum muscle, which is localized posteriorly, was stripped off and spinous processes and transverse processes were exposed. Spinal fusion was performed in L4 to L6. Spinous processes of the lumbar vertebrae were taken off by rongeur and bones were cleared of their soft tissues. Transverse processes were decorticated. The lumbar region in which the graft was placed was decorticated with rongeur, curette, and thin burr. In Groups I and III, grafts were applied without amniotic fluid (ie, allograft only in Group I and DBM only in Group III). In Group II and Group IV, in addition to allograft or DBM, 0.5 cc processed amniotic fluid was applied to the posterior spinal elements that were decorticated following fusion with grafts.

During the first 7 days of postsurgical follow-up, wound dressings and examination of the surgical site were performed. Immobilization was not implemented. Rats were killed at Week 8 using high-dose ether anesthesia. Cervical dislocation was not performed on rats because it could affect the fusion. The fusion line was accessed through a posterior midline incision involving the skin, subcutaneous tissue, and muscle layer. The fusion area was carefully dissected with bone scissors avoiding injury to the fusion site at its proximal and distal ends. The removed segments with fusion were placed in bottles containing 10% formaldehyde.

Radiologic evaluations

Each fusion segment extracted was assigned a number and placed on a 30 × 24 cm radiograph cassette so that tube distance was 90 cm. Then anteroposterior radiographs digitally shot were evaluated. Radiographic images were evaluated by a single radiologist who was blinded to the type of grafting implemented. Radiologic assessments were based on Lenke's radiologic evaluation system[9] using a 1- to 4-point scale, where 1 = bilateral graft resorption or fusion mass with obvious bilateral pseudarthrosis; 2 = bilateral small, thin fusion masses; 3 = unilateral large fusion mass with contralateral small fusion mass; and 4 = solid large trabeculated bilateral fusion masses.

Histologic examinations

Samples from which radiologic images were obtained after the rats were killed were decalcified in 10% formic acid at room temperature for 80 days. Decalcification solution was changed every 3 days during this period. Samples were dehydrated with ethanol, cleaned with xylene, and buried in paraffin. Longitudinal sections of 5 μm were done by microtome knife and hematoxylin-eosin stain was applied. All cross-sections were evaluated with a light microscope (Olympus BX-51, Postfach, Hamburg, Germany) at the histology laboratory and microphotographs were obtained. A 0- to 7-point scaling system described by Emery et al[10] was used for histopathologic evaluations, where 0 = empty cleft, 1 = fibrous tissue only, 2 = more fibrous tissue than fibrocartilage, 3 = more fibrocartilage than fibrous tissue, 4 = fibrocartilage only, 5 = more fibrocartilage than bone, 6 = more bone than fibrocartilage, and 7 = bone only.

Statistical analyses

Statistical evaluation was done using SPSS 13.0 for Windows (IBM-SPSS Inc, Armonk, NY). Differences between the groups in terms of scores were analyzed using Kruskal-Wallis variance analysis and Mann-Whitney *U* test was used for pairwise comparisons. A P value < 0.05 was considered an indication of statistical significance for the variance analyses. For post-hoc analyses, Bonferroni correction was made and the level of significance was set at $P < 0.0083$ for Mann Whitney *U* test.