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Research Article

The Histopathological Evaluation of Effects of Application of the Bovine Amniotic Fluid with Graft on Peri-Implant Bone Regeneration

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ABSTRACT

This study aimed to determine the effects of bovine amniotic fluid combined with bone graft in treating peri-implant bone defects with guided bone regeneration. Twenty female Sprague–Dawley rats were divided into two groups. Bone sockets with a diameter of 4 mm in the coronal part and a diameter of 2.5 mm in the apical part of the implant were created into the corticocancellous bone in the metaphyseal parts of the right tibia bones of all subjects. Implants with a length of 4 mm and a diameter of 2.5 mm were placed in the bone sockets. In the sham surgery group (n = 10) was the circumferential bone defect equivalent to half of the 4-mm implant length, which occurred between the implant and the bone, filled with bovine xenograft. Bovine xenografts were filled with amniotic fluid mixture in the experimental group (n = 10). After 8 weeks of recovery, all rats were sacrificed. The implants were extracted from the soft tissues and the surrounding bone. Subsequently, the bones were decalcified and prepared for histological analysis. The percentage of newly regenerated bone (NRB) formation and fibrosis in the bone defect area around the implant was calculated from all sections. NRB was found in 37.4±4.4% of controls and 41.4±2.63% of test animals ($P<0.05$ and $P=0.024$, respectively). Fibrosis formation was found at a rate of 38.6±5.06% in the control group and 33.2±5.38% in the test group ($P<0.05$ and $P=0.033$, respectively). It was considered that combining bovine amniotic fluid with bone transplant could be a useful way of treating bone abnormalities.

Keywords: Bone graft, Bovine amniotic fluid, Guided bone regeneration, Peri-implant bone defect, Tibial bone

Introduction

Titanium-manufactured implants have recently become one of the most popular options in orthopedics, oral and maxillofacial surgery, and prosthetic dentistry [1]. With their functionality, aesthetics, ease of use, and comfort, titanium-made implants are a trustworthy material in fracture treatment [1,2]. Successful treatments with titanium implants in orthopedic and maxillofacial implant operations depend on the extent of osseointegration, which is described as direct contact of the bone with the titanium implant surface, as well as the properties of the implant material. In this regard, bone formation and regeneration mechanisms are important parameters for effective osseointegration [3].

Guided bone regeneration (GBR) applications have proven to be effective in treating abnormalities and deficiencies in peri-implant bone tissue. In the presence of bone defects and insufficient bone tissue, GBR is necessary for 40% of patients with implants to ensure healthy osseointegration. The survival rates of patients with GBR-treated implants have been comparable with those of non-GBR-treated implants. Titanium implants have a high survival rate after GBR treatment and a good survival rate after functional loading. Studies demonstrating a 95% implant survival rate after horizontal or vertical GBR treatment with

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various bone graft materials have been recorded in the literature. In this regard, GBR is a scientifically approved and successful treatment method frequently used with various bone graft materials, including otogen, allogeneic, xenogeneic, and alloplastic, for treating bone tissue defects around the titanium implant [1-4].

Stem cells were obtained from amniotic fluid and these stem cells attracted the attention of researchers. Amniotic mesenchymal stem cells can be isolated from amniotic fluid. Therefore, their use does not fall within the ethical concerns associated with the use of embryonic stem cells. Furthermore, like other fetal-derived stem cells, amniotic mesenchymal stem cells are easy to store and can be obtained at a low cost. Populations of amniotic mesenchymal stem cells can be easily grown, and these cells can be kept for a long time without any adverse effects. Therefore, amniotic fluid can be considered a source of pluripotent and multipotent stem cells for organ regeneration [5-8].

Bovine amniotic fluid contains various proteins, minerals, and cells [8]. Cattle have a well-developed allantoic cavity containing abundant amniotic fluid. Because of their nature, they can be a valuable source for obtaining amniotic fluid to treat various diseases [8]. Unlike embryonic stem cells, amniotic fluid stem cells do not develop teratomas when injected subcutaneously into mice [9-12]. Therefore, amniotic fluid stem cells are considered an intermediate stem cell type as they exhibit embryonic and adult stem cell characteristics. Wound repair and regeneration can be aided by amniotic tissue products [13].

Amniotic fluid contains high concentration of various growth factors that may be involved in bone formation mechanisms [9-14]. This study examined the efficacy of bovine amniotic fluid in combination with bone graft in the regeneration of experimentally created tibia peri-implant bone defects in rats.

Material and Methods

Ethical Approval

The study's experimental and surgical methods were implemented in the experimental research center of Fırat University Elazığ, Turkey. Before the experimental applications were carried out, ethical approval was obtained from the Fırat University Animal Experiments Local Ethics Committee (Protocol Number: 24/02/2020-380123). Rats were cared for and kept in their special cages in accordance with the Declaration of Helsinki.

Animal and Study Design

In the study, 20 female Sprague-Dawley rats were divided into two groups. In the control implant + graft group (n = 10), titanium implants 4 mm long and 2.5 mm in diameter were applied to the tibia bones of the rats. Titanium implants were inserted into implant beds developed in the corticocancellous bone tissue of the right tibia bones of the rats. A bovine bone graft was implanted in the circumferential bone defect surrounding the implants, accounting for 2 mm of implant length. During the 8-week healing period, no additional procedures were performed on the rats in this group. Following decalcification, the bone tissue containing implants was separated from the soft tissues and subjected to histological analysis. The percentages of new bone development and fibrosis in the defect around the implant were calculated [3,4]. In the implant + graft + amniotic fluid group (n = 10), titanium implants with 2.5 mm diameter and 4 mm length were applied to the tibia bones of the rats. The implants were inserted in the corticocancellous bone tissue of the rats' right tibial metaphyseal bones, and amniotic fluid and bovine bone graft were combined and placed in the bone defect created in the neck region, corresponding to 2 mm of the implant length. Bone grafts were obtained by mixing 2 mL bovine amniotic fluid with 1 mL bone graft. During the 8-week study period, no experimental procedures were used. Rats were sacrificed after an 8-week experiment. After removing the soft tissues, the implants and surrounding bone tissues were collected and histologically analyzed. The percentages of new bone formation and fibrosis in the defect around the implant were calculated [3,4].

The area of the peri-implant defect was initially measured before assessing new bone growth and fibrosis. The regions of new bone tissue and fibrosis that had grown within the defect were measured. The percentages of new bone formation and fibrosis were calculated by dividing the new bone tissue and fibrosis values by the defect area [3,4].

Obtaining Bovine Amniotic Fluid

During cesarean delivery at the Fırat University Animal Hospital, bovine amniotic fluid was taken from a healthy pregnant cow using an injector. The sample was brought to the laboratory in an ice box without breaking the cold chain, and it was stored in a deep freezer at −20°C until the day of surgery. It was used after allowing the sample to dissolve at room temperature for 15 min.

Surgical Procedures

Following general anesthesia, all surgical applications were performed under sterile conditions. General anesthetics (10 mg/kg xylazine HCl [Rompun®, Bayer, Germany] and 40 mg/kg ketamin HCl [Ketasol®, Richter Pharma, Germany]) were administered intraperitoneally using a rat-specific injector. During the surgical application preparation phase, the surgical area was shaved and then cleansed with povidone-iodine. An incision was made

over the right tibia bones of the rats with a no. 11 scalpel during the surgical implantation of the implants, and the metaphyseal part of the bone was reached. The surgical region was cleansed with physiological saline to prevent warmth while the implant sockets were opened. A hollow with a width of 4 mm from the coronal part and a diameter of 2.5 mm in the apical half was opened to half the length of the implant while forming the implant sockets. Following implant placement, the circumferential bone defect of 1.5-2 mm developed surrounding the implant was filled with bovine bone graft in the sham surgery group and amniotic fluid mixture with bovine bone graft in the test group [3,4]. After surgical insertion, the soft tissues were sutured with a 4-0 absorbable suture (polyglactin). Antibiotics (50 mg/kg Penicillin [Ampisina®, Gensenta İlaç San. Tic. A.Ş, Turkey]) and analgesics (0.1 mg/kg Tramadol hydrochloride [Ramadex®, Haver Trakya İlaç San. Tic. A.Ş, Turkey]) were administered intramuscularly for 3 days following all surgical operations to avoid infection and pain. Whole rats were slaughtered after 8 weeks of surgical procedures [3,4].

Histological Analysis

After removing the soft tissues, titanium implants and surrounding bone were collected for histomorphometric analysis. All bone samples were preserved in 10% formaldehyde for tissue fixation. After that, the bone tissues were softened in 10% formic acid. The implants were gently removed from the bone after the surrounding bone tissues relaxed. After drying, the softened samples were embedded in paraffin wax. Before microscopic analysis, all samples were stained with hematoxylin, eosin, and Masson trichrome. Histological assessment was performed using a light microscope. A light microscope was used to examine bone tissue sections with a total thickness of 6 µm surrounding the implants. The percentage of bone filling and intradefect fibrosis was evaluated [1-3].

Statistical Analysis

The Student's t-test was used to determine whether there was a significant difference in the experiment data (due to the availability of two more test groups not stated in this article). All values included in the statistical phase of the study were expressed as mean ± standard deviation (SD), and P<0.05 was considered the statistically significant value.

Results

Table 1 shows the distribution of newly regenerated bone (NRB) and fibrosis among groups. The graft with the bovine amniotic fluid group had the highest NRB, whereas the sham control group had the lowest. The mean values of NRB in the BAF graft and sham control groups

Table 1. *Histologic new bone formation (NRB) an fibrosis parameters of the groups after the H&E eosin and masson trichrome staining*

Parameters	Groups	N	Mean (%)	SD	P*
NRB	Amnion	10	41.4	2.63	0.024
	Control	10	37.4	4.4	
Fibrosis	Amnion	10	33.2	5.38	0.033
	Control	10	38.6	5.06	

** Student T Test (p<0.05). Statistically significant difference detected between the groups*
NRB: Newly Regenerated Bone, SD: Standard Deviation

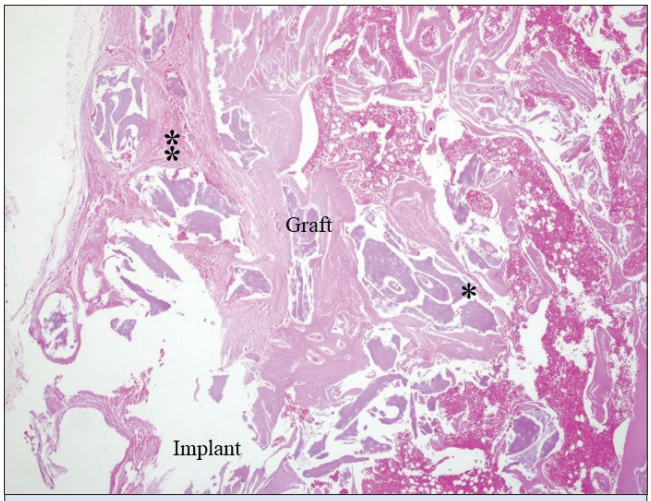

Fig 1. Decalcified histologic images of the sham control group (4 *g* magnification, Hematoxylin-Eosin). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. * Newly regenerated bone, ‡ Fibrosis

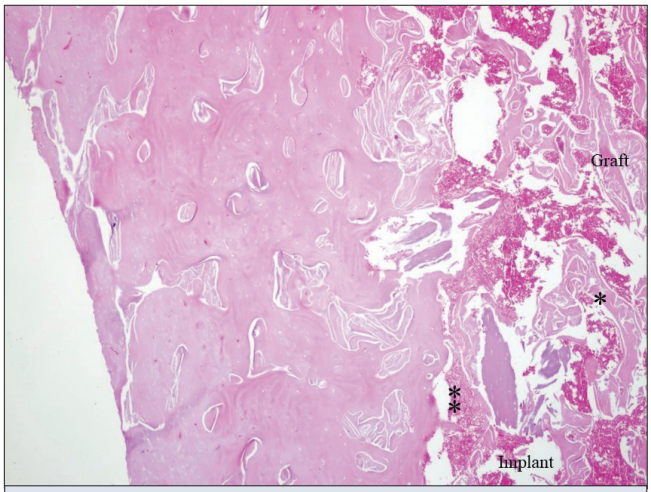

Fig 2. Decalcified histologic images of the bovine amniotic fluid and graft mixation group (4 *g* magnification, Hematoxylin-Eosin). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. Bone filling and maturation of the defect in the experimental group were statistically higher than in the sham group. * Newly regenerated bone, ‡ Fibrosis

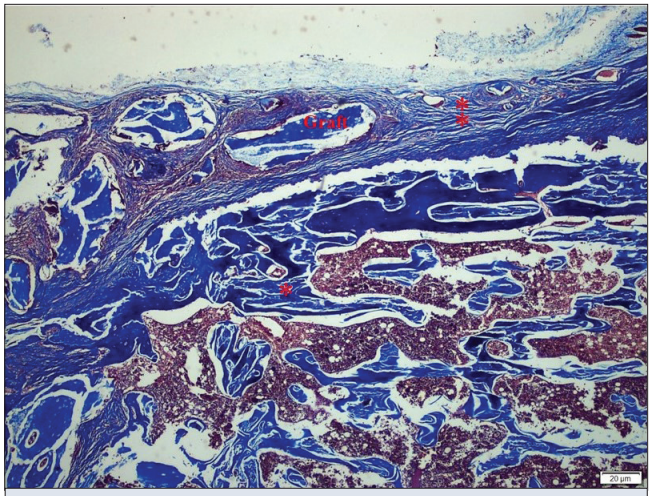

Fig 3. Decalcified histologic images of the sham control group (10 *g* magnification, Masson Trichrome). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. * Newly regenerated bone, ‡ Fibrosis

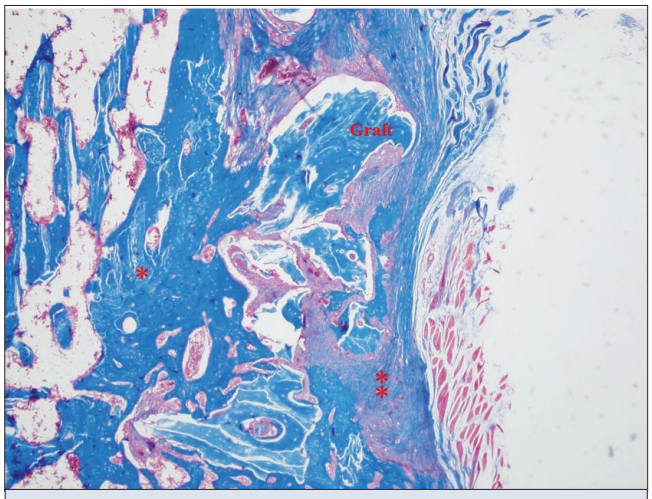

Fig 4. Decalcified histologic images of the bovine amniotic fluid and graft mixation group (10 *g* magnification, Masson Trichrome). Bovine xenografts appeared to ossify at the implant neck defect site and are surrounded by fatty bone marrow. Ossification areas are surrounded by fibrosis. Bone filling and maturation of the defect in the experimental group were statistically higher than in the sham group. * Newly regenerated bone, ‡ Fibrosis

were 41.4±2.63% and 37.4±4.4%, respectively (P<0.05 and P=0.024). The graft with the bovine amniotic fluid group had the highest fibrosis level, whereas the lowest in the sham control group had the lowest. The mean values of fibrosis in the bovine amniotic fluid graft and sham control groups were 33.2±5.38% and 38.6±5.06%, respectively (P<0.05 and P=0.033). There were statistically significant differences between groups in NRB and fibrosis values (*Fig. 1*, *Fig. 2*, *Fig. 3* and *Fig. 4*).

Discussion

Bovine amniotic fluid is a relatively new material, and research on its effects on bone metabolism is ongoing. Positive effects of bovine amniotic fluid on bone tissue, tendon, and nerve regeneration have been reported in the literature [15,16]. Furthermore, some researchers have found that bovine amniotic fluid is less expensive and easier to obtain than human amniotic fluid. Mesenchymal cells, insulin growth factors, other growth factors, and macromolecules, such as hyaluronic acid and hyaluronic acid stimulating factor, are found in bovine amniotic fluid [17,18]. The increased percentage of newly formed bone tissue in the bone defects and lower fibrosis levels reported in the participants transplanted with bovine amniotic fluid are assumed to be to these characteristics of bovine amniotic fluid.

This study aims to generate new perspectives for improving the rate of bone formation and bone quality. Bovine amniotic fluid is a successful method in GBR with bone graft. The components of bovine amniotic fluid have allowed it to be used in various applications [19]. Insulin-like growth factor, for example, can be actively produced by the placenta of ruminant animals [20]. Insulin-like growth factor-1 can directly and indirectly increase osteoblast matrix production [21]. Therapeutic applications, such as growth factors and other regenerative agents, may be required to enhance bone formation around implant surfaces. Furthermore, an osteogenic coating on implant surfaces has been proposed to improve osseointegration and bone tissue regeneration around the implant [22]. Therefore, using bovine amniotic fluid is believed to play a role in increasing GBR around the implant. Previous studies reported that amniotic fluid stem cells activated the bone morphogenic protein gene [23]. The bone morphogenic protein is involved in regeneration by generating the signal that causes osteoblast maturation. Bone morphogenetic proteins are the most critical growth factors for bone formation and repair among bone-related growth factors [24,25]. Osteoblast maturation is also initiated by bone morphogenetic protein [26]. Bovine amniotic fluid can be considered a diverse supply of mesenchymal cells. These mesenchymal cells can develop into various cell types, including adipose, muscle, bone, and neural lineages [24-26].

In this study, GBR was performed, a procedure commonly used to treat bone defects around the implant and has gained popularity in the literature. The data gathered at the end of the trial confirmed that the biological ingredients in bovine amniotic fluid, when used in conjunction with the graft to repair bone defects, could yield more beneficial results. In the presence of insufficient bone tissue, bone grafts, commonly used in oral-maxillofacial surgery, periodontology, oral implantology, and orthopedic surgery, are an essential aspect of the treatment. In the present literature, autogenous bone grafts are still considered the gold standard. However, due to difficulty in

acquiring autogenous grafts, defects in the donor location, neurovascular injuries in obtaining autogenous grafts, and early resorption, clinicians typically use animal-derived xenografts, synthetic alloplastic grafts, and human-derived allogeneic grafts [1-4,5,7]. This study examined bone healing between xenograft mixed with bovine amniotic fluid and the xenograft alone group. This study hypothesized that biological components can accelerate bone healing, such as hyaluronic acid, mesenchymal stem cells, and growth factors in bovine amniotic fluid. The xenograft was combined with enough bovine amniotic fluid to make it plasmatic, and the circumferential bone defect around the implant was filled [1,3,4,15,16,24-26].

The bovine xenografts used in the study were freeze-dried and obtained through various laboratory processes, have reduced antigenic properties, and are commonly used therapeutically clinically in orthopedic and oral maxillofacial surgery. Furthermore, the use of rats in this study was indicated by the study by Ozgenel et al.[16] They used macroscopic and histological methods to compare nerve recovery using human amniotic fluid in sciatic nerve injury with controls and found no immunogenic response in rats in the human amniotic fluid group.

This study evaluated bovine amniotic fluid's effectiveness in guided bone regeneration when used with xenograft using a histological approach. Bovine amniotic fluid, which contains chemicals such as mesenchymal stem cells, hyaluronic acid, and growth factors, has become a material that can positively influence bone tissue healing. The findings of this study suggest that using bovine amniotic fluid in conjunction with bone transplant may be an effective method in GBR, which is used in treating peri-implant bone defects.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (Ozmen Istek) at the University of Mus Alparslan University, Faculty of Health Sciences, Department of Nursing, Mus, Turkiye.

Funding

There is no funding.

Conflict of Interest

There is no conflict of interest.

Ethical Approval

Ethical approval was obtained from the Fırat University Animal Experiments Local Ethics Committee before the experimental applications were carried out (Protocol Number: 24/02/2020-380123).

Author Contributions

O.I., M.T., and S.D. designed the study. O.I., S.D., and M.T. did the experimental procedures. H.E. and B.K. did the histological procedures. B.K., S.D., M.T., and H.E. did the histological analysis. S.D. did the statistical analysis. O.I., R.G., E.C.O., S.D., and M.B.B. wrote the study. R.G., M.B.B., and E.C.O. controlled the manuscript.

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