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Evaluation of the effect of local Bovine Amniotic Fluid on Osseointegration of Titanium Implants: A Histologic and Histomorphometric Study

Evaluación del efecto del líquido amniótico bovino local sobre la osteointegración de implantes de titanio: un estudio histológico e histomorfométrico

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ABSTRACT

The aim of this study was to histologically and histomorphometrically investigate the effect of locally applied bovine amniotic fluid (BAF) on osseointegration levels in implants. Adult female Sprague–Dawley rats weighing 300–350 g were used as subjects. The rats were divided into two groups: the sham–operated control group (n=10) and the local BAF group (n=10). Implant cavities were created in the tibias of all subjects under sterile saline cooling with rotating instruments. Local BAF was applied to all implant sockets before the implants were placed. Rats were sacrificed after a four-week osseointegration period. Histological staining was performed using hematoxylin and eosin staining to analyze the osseointegration. Examinations of the bone implant connection (BIC) and peri-implant bone formation (PBF) were performed using a light microscope and an image analyzer. As a result of the analysis, the mean BIC value was 40.3 ± 4.9 for the sham–operated control group and 45.2 ± 7.7 for the local BAF group. The mean PBF was 39.9 ± 6.3 for the sham control group and 40.5 ± 5.7 for the local BAF group. A statistically significant difference was found between the sham control group and the local BAF group for the BIC and PBF values (*P*>0.05; *P*: 0.11; *P*: 0.83). The application of local BAF to the implant socket did not have a clear positive effect on implant osseointegration. More studies are needed to clarify the association between local BAF and osseointegration.

Key words: Bovine amniotic fluid; titanium implant; osseointegration; bone implant connection; bone implant contact

RESUMEN

El objetivo de este estudio fue investigar histológica e histomorfométricamente el efecto del líquido amniótico bovino (BAF) aplicado localmente sobre los niveles de osteointegración en implantes. Se utilizaron como sujetos ratas Sprague–Dawley hembras adultas que pesaban entre 300 y 350 g. Las ratas se dividieron en dos grupos: el grupo de control con operación simulada (n=10) y el grupo BAF local (n=10). Se crearon cavidades para implantes en las tibias de todos los sujetos bajo enfriamiento con solución salina estéril con instrumentos giratorios. Se aplicó BAF local a todos los alvéolos de los implantes antes de colocarlos. Las ratas fueron sacrificadas después de un período de osteointegración de cuatro semanas. La tinción histológica se realizó mediante tinción con hematoxilina y eosina para analizar la osteointegración. Los exámenes de la conexión ósea–implante (BIC) y la formación ósea periimplantaria (PBF) se realizaron utilizando un microscopio óptico y un analizador de imágenes. Como resultado del análisis, el valor BIC medio fue de 40,3 ± 4,9 para el grupo de control con operación simulada y de 45,2 ± 7,7 para el grupo BAF local. El PBF medio fue 39,9 ± 6,3 para el grupo de control simulado y 40,5 ± 5,7 para el grupo BAF local. Se encontró una diferencia estadísticamente significativa entre el grupo de control simulado y el grupo BAF local para los valores de BIC y PBF (*P*>0,05; *P*: 0,11; *P*: 0,83). La aplicación de BAF local al alvéolo del implante no tuvo un efecto positivo claro sobre la osteointegración del implante. Se necesitan más estudios para aclarar la asociación entre BAF local y la osteointegración.

Palabras clave: Líquido amniótico bovino; implante de titanio; osteointegración; conexión implante óseo; contacto implante óseo

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INTRODUCTION

Amniotic fluid is the clear, watery fluid surrounding the growing fetus in the amniotic cavity, and it allows the fetus to grow and move freely in the womb, softening sudden impacts or movements, protecting it from external influences, and allowing the exchange of chemicals between mother and fetus [1]. Bovine amniotic fluid (BAF), a form of amnion fluid found in cattle, consists of proteins, minerals, and cells, whose relative amounts can change during pregnancy [2]. It also contains carbohydrates, fats, amino acids, enzymes, hormones, and pigments [3]. In addition to these properties, BAF contains insulin-like growth factor (IGF) and other growth factors that have a stimulating effect on mesenchymal cells [4]. Moreover, cells derived from this fluid have been found to have a cell marker profile comparable to mesenchymal stem cells, and they differentiate into osteocytes, adipocytes, and fibroblasts [5]. Previous studies have shown that growth factors in amniotic fluid play an important role in human embryo growth and development [6].

This evidence shows that, after proven safety and definitive efficacy, large-animal models may be a reasonable choice for translational studies in humans [7, 8].

Cattle (*Bos taurus*) are an important species of commercial value and are an attractive large-animal model for biomedical research [9]. Cattle also have a well-developed allantoic cavity, with a large amount of amniotic fluid. Therefore, they can be a useful source of amniotic fluid for human diseases [2]. In addition to these advantages, the risk of miscarriage associated with the collection of human amniotic fluid, although low (1 in 200), is still a serious ethical concern.

Osseointegration is defined as a direct structural and functional connection between bone tissue and the implant surface. The use of titanium endosseous dental implants in the rehabilitation of partially or fully edentulous patients is an alternative for restoring function and aesthetics [10]. In general, implant success rates reach 90% 10–15 years after implantation [11, 12]. Therefore, given the large number of titanium dental implants placed each year, a 10% failure rate translates into millions of failure cases [13]. Despite the high success rates provided by implants, studies have been carried out to accelerate osseointegration with different technologies and manufacturing methods [14]. Based on this ambition, studies have reported that hormone replacement therapy, bisphosphonates, and intermittent administration of parathormone improve bone quality around implants in subjects [10].

By increasing the bone implant connection (BIC) and peri-implant bone formation (PBF), additional treatments, such as vitamin D supplementation and hormone replacement, have been reported to improve the success and survival rates of dental implants [15]. Likewise, studies evaluating the effectiveness of parathormone and melatonin supplementation on the osseointegration of implants have found promising results in animal models, but more research is needed to evaluate their effectiveness in human [16, 17].

Considering the regenerative properties of BAF, the present study aimed to examine the effect of BAF, an inert fluid rich in growth factors, on bone healing and bone implant fusion.

MATERIALS AND METHODS

Animals and study design

All of the experimentation procedures were approved by the Animal Experiments Local Ethics Committee at the University of Firat (Date: February 24, 2020; Protocol no: 380123). The experimental animals were obtained from the Firat University Experimental Research Center. The study was conducted at the Firat University Experimental Research Center operating rooms.

In the current experimental study, 20 machined-surface titanium implants (Implance Dental Implant System, AGS Medical Corporation, Istanbul, Turkey) with a diameter of 2.5 mm and a height of 4 mm were used. The cleaning process was completed by autoclave.

This study was carried out using 20 healthy female Sprague-Dawley rats (*Rattus norvegicus*) aged six months. To ensure standardization of the study, vaginal smears were performed, and rats in the same estrus period were included in the study. The Sprague-Dawley rats were divided into two groups (10 rats each): the control group (implant alone) and the test group (implant with applied BAF).

For the control group (n=10), bone osteotomies were performed at the corticocancellous bone in the tibial metaphysis, and machined-surface titanium implants of 2.5 mm in diameter and 4 mm in length were installed in each rat with no additional treatment.

For the test group (n=10), BAF was locally applied in the implant sockets before the machined-surface implants were placed in each rat. During the experimental period, no additional treatment was applied. After four weeks, the subjects were sacrificed. Implants and the surrounding bone tissue were taken and subjected to histological analysis after decalcification.

Collection of bovine amniotic fluid (BAF)

During cesarean delivery in Fırat University Animal Hospital, BAF was taken from a healthy pregnant cow under aseptic conditions with an 20 mL injector (ClickZip,Medical Device Manufacturer, Thailand). This amount was immediately dispensed into 2 mL syringes (ClickZip,Medical Device Manufacturer, Thailand) in a cold environment and on an icebox for easy handling. The BAF was brought to the laboratory in an ice box without breaking the cold chain and stored in a deep freezer (Arçelik, 2533D, Türkiye) at -20°C until the day of the operation. It was used after being allowed to dissolve at room temperature for 15 min.

Surgical procedures

Before the surgical experiments, the rats were anesthetized with an injection of Ketamine hydrochloride (50 mg·kg^{-1}, Ketasol; Richter Pharma, Wels, Austria) and Xylazine (5 mg·kg^{-1}, Rompun; Bayer, Germany). Before surgery, the shaved skin of the leg was carefully washed with sterilized iodine. Then, a full-thickness longitudinal incision was made in the upper-middle region of the back right tibia, and the area was revealed for experimentation. 2.5 mm diameter and 4 mm length monocortical bone cavities were created by drilling the corticocancellous bone of the tibial metaphysis. Meanwhile, a significant amount of sterilized physiological saline solution was used to irrigate the area for cooling purposes. 2.5 mm diameter and 4 mm length titanium implants were then inserted through the locating region. The surgical region was then closed with 4-0 silk sutures, and the rats were allowed to recover. After the surgical procedures, an antibiotic (40 mg·kg^{-1} Cefazolin sodium) and an analgesic (Tramadol hydrochloride 0.1 mg·kg^{-1}) were administered intramuscularly to the rats.

Histomorphometric measurements

At the fourth week after implantation, the rats were scarified with full anesthesia. The tibias were dissected, and the implants were

extracted with nearby bone tissue, thus allowing blocks containing the experimental specimens to be obtained. The specimens were immediately immersed in 10% formalin and fixed at room temperature.

Osseointegrated implants and the surrounding bone tissue were used for the histomorphometric analysis. When first taken, all samples were kept in 10% formaldehyde for the fixation of the tissue. The bone tissue was then transferred to 10% formic acid to soften it. After the surrounding tissue softened, the implants were carefully removed from the bone. The softened samples were dried and embedded in paraffin wax. Finally, they were prepared for microscopic (Olympus BX43, Tokyo, Japan) examination by staining with hematoxylin and eosin. Bone tissue sections with a total thickness of 6 µm surrounding the implants were taken and evaluated with a light microscope (Olympus BX43, Tokyo, Japan). Hematoxylin and eosin staining was preferred because it is a reliable application that is frequently used in the literature. In addition, hematoxylin and eosin can be used safely in measurements regarding the interaction of bone tissue with the implant surface.

To analyze the bone tissue, histological staining was performed using hematoxylin and eosin staining. BIC and PBF (Peri-implant bone formation) examinations were performed using a light microscope and an image analyzer (Olympus DP72, Tokyo, Japan) in the laboratory at the Firat University Department of Pathology, Faculty of Medicine.

The histomorphometric analysis was measured using a software program (Olympus, DP71, Japan). PBF and BIC examinations were performed by the same expert using stereological software. The BIC evaluation analyses were determined by the ratio (%) of the surface part of the implant in contact with the bone to the entire implant surface [10, 18, 19]. The PBF ratio was evaluated as the ratio of the bone formed in the area around the implant and was calculated separately for each sample in a region of interest created at a distance of 0.5 mm from the mesial, distal, and apical edges of the implants (FIG. 1)[19]. The researchers used the Olympus DP71 imaging software for the histomorphometric analysis.

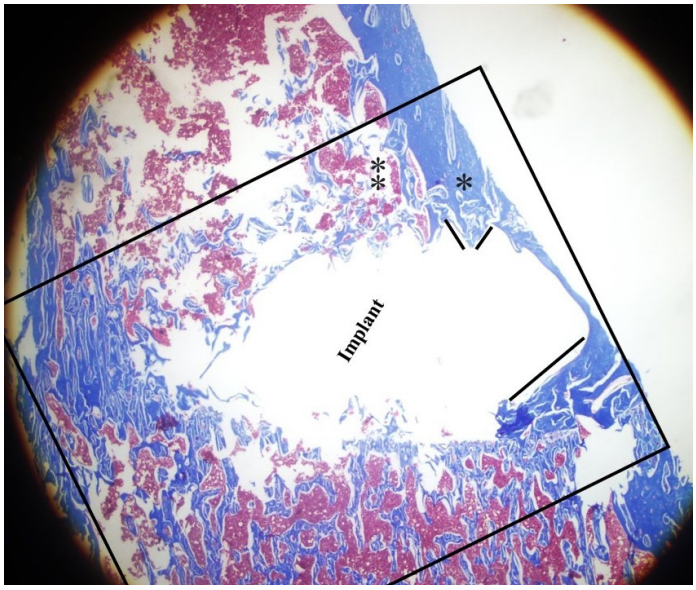

FIGURE 1. Black line: Bone implant connect lenght, *: Periimplant bone. ‡: Bone marrov. Bone Implant Connection Ratio (%): Implant surface contacting bone / Total implant surface. PBF (Peri-implant bone formation): Total bone area/ Region of interest area.

Statistical analysis

The significant difference in the experimental data was analyzed with the Student's ttest after Shapiro-Wilks and Kolmogorov-Smirnov tests were conducted (due to the existence of two groups and the parametric data)(IBM SPSS Statistics 22 Program). All values involved in the study were denoted as mean ± standard deviation; $P<0.05$ was accepted as the value of statistical significance.

RESULTS AND DISCUSSIONS

TABLE I shows the BIC values of the two groups. A higher BIC value was found in the local BAF group than in the sham control group. The mean values of BIC in the local BAF and sham control groups were 45.2 and 40.3%, respectively. In the treatment group, high osseointegration levels of the BIC were detected, compared to the controls, but this was not statistically significant ($P>0.05$; $P=0.11$). TABLE I also shows the PBF values of the groups. A higher PBF values was found in the local BAF group than in the sham control group. The mean PBF value in the local BAF and sham control groups were 40.5% and 39.9%, respectively. No significant difference was found for the PBF values between the groups ($P>0.05$; $P=0.83$).

TABLE I
Histologic BIC and PIB of the groups after the hematoxylin-eosin staining

Parameters	Groups	N	Mean	Std. Dev.	P*
BIC (%)	Amnion	10	45.2	7.7	0.11
	Control	10	40.3	4.9	
PBF (%)	Amnion	10	40.5	5.7	0.83
	Control	10	39.9	6.3	

$P>0,05$; $P_1=0,11$, $P_2=0,83$. * Student *t* Test. Statistically significant differences was not detected between the groups. BIC: Bone implant contact. PBF: Periimplant Bone Formation

When the histological sections were examined, bone apposition was seen around the implants. Bone formation occurred according to the thread shapes of the implants, but no inflammation was detected in the parts where the implant and bone tissue came into contact. However, all of the analyzed implants were osseointegrated. Fatty bone marrow in the bone tissue of the sections can be seen in FIGS. 2 and 3.

Osseointegration, which involves the interaction between the bone tissue and the implant, affects the long-term success of implants [18]. High levels of osseointegration play an important role in the long-term survival and success of implants [16]. Therefore, one of the subjects of academic and commercial interest in this field is to discover methods that stimulate and accelerate osseointegration [19]. In determining the success of the osseointegration process, histological evaluation and histomorphometry can be performed by examining peri-implant bone tissue in experimental animals [18].

Additional stimulants are necessary to increase osteogenesis, especially to overcome failures in poor bone quality and thus shorten the loading time of dental and orthopedic implants [20]. Studies have reported that adjunct treatments (e.g., the use of growth factors and/or stem cells, hormone replacement, and vitamin D supplementation) in conventional implant placement protocols play a role in enhancing BIC, which may, in turn, improve the overall success and survival of

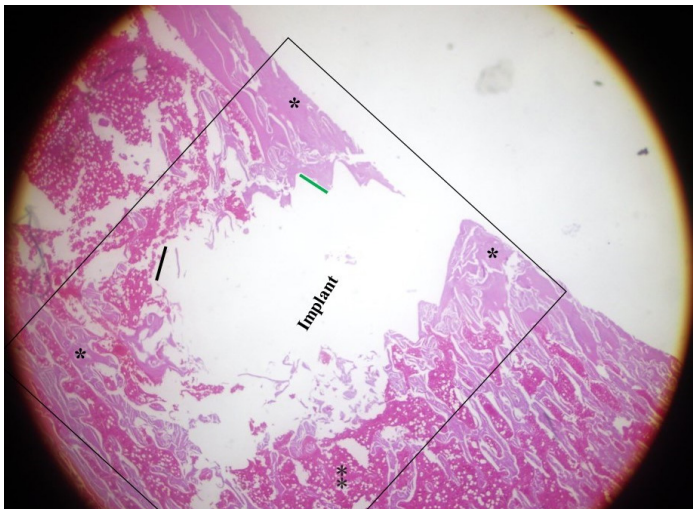

FIGURE 2. Decalcified histologic images of the local Bovine Amniotic Fluid Group (4×, ×:10, hematoxylin–eosin, *: Bone Tissue). Black line: non–bone connect area. Green Line: Implant surface connecting bone. Bone Implant Connection Ratio (%): Implant surface contacting bone / Total implant surface. PBF (Peri–implant bone formation): Total bone area/ Region of interest area

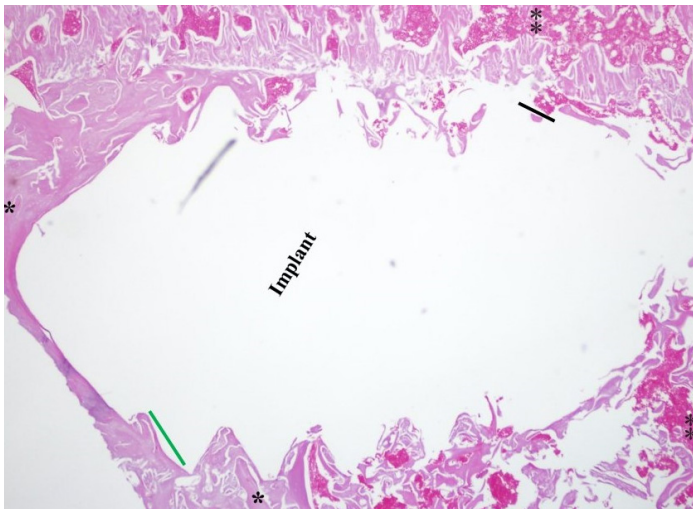

FIGURE 3. Decalcified histologic images of the Sham Control Group (4×, ×:10, hematoxylin–eosin, *: Bone Tissue). Black line: non–bone connect area. Green Line: Implant surface connecting bone. Bone Implant Connection Ratio (%): Implant surface contacting bone / Total implant surface. PBF (Peri–implant bone formation): Total bone area/ Region of interest area

dental implants [15]. Similar to the results of these studies, laminin coatings on implant surfaces promote osseointegration [21].

Despite many research results, initial osseointegration and rapid bone healing are still important issues in the clinical field [22]. Data from experimental studies investigating and comparing the effects of BAF on bone healing in implant osseointegration are scarce. Therefore, based on preclinical studies evaluating the effects of BAF on wound healing and bone metabolism, this study aimed to investigate the effect of BAF, an inert fluid rich in growth factors, on osseointegration in the tibias of rats through descriptive histological and histomorphometric analyses.

Many studies have suggested different applications of BAF due to its contents, such as IGF, which is actively synthesized by the placentas of ruminant animals [2, 23]. Osteoblast matrix synthesis can be stimulated directly and indirectly by IGF–1 [19]. To increase bone formation around the implant surfaces, therapeutic methods, such as the use IGF–1 of other growth factors (e.g., platelet–derived growth factor, basic fibroblast growth factor, and bone morphogenetic protein 2), and osteogenic coating on implant surfaces, have been suggested [21]. It is therefore hypothesized that using BAF can play a role in enhancing osseointegration, as previous studies have shown that the bone morphogenic protein (BMP) gene is activated by amniotic fluid stem cells [18, 24]. BMP signaling that initiates osteoblast maturation can be counted, even among bone–related growth factors, as the most important growth factor for bone formation and healing [25]. Amniotic fluid is known to contain a heterogeneous population of cell types whose derived cells can give rise to a variety of differentiated cells, including adipose, muscle, bone, and neuronal lineages.

Considering the results of the analysis, although no significant superiority was determined between the test and control groups in terms of BIC and PBF, increases in both BIC and PBF levels were detected. This may be due to the regenerative capacity of BAF.

In a similar study, Istek *et al.* examined guided bone regeneration around implants and reported that BAF used locally with xenografts increased bone regeneration in peri–implant bone defects with statistical significance compared to controls [26].

Istek *et al.* reported that this result may be due to the fact that BAF, which contains biochemicals, such as mesenchymal stem cells, hyaluronic acid, and growth factors, increases the healing of bone tissue [18, 20, 26].

In additon to this Tanrisever *et al.* reported that local bovine amniotic fluid application may positively affect the healing of bone fractures. In their study, they examined the effect of local amniotic fluid on bone healing in experimentally created fractures in rat tibias using histopathological methods [27].

CONCLUSION

Based on the limited results of this study, it can be stated that local BAF application does not have an effect on implant bone connection. In this work, an approach for increasing the osseointegration levels of dental implants was proposed. From a preclinical perspective, further experimental studies are needed to assess the role of using BAF in promoting osseointegration around dental implants.

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Ethics approval and consent to participate

The present study was performed in line with the principles of The Declaration of Helsinki. Approval was granted by the Fırat University Experimental Animal Ethics Committee (24 February 2020, Protocol no: 380123; Elazığ, Turkiye).

Conflict of interest

The authors declare there is no conflict of interest.

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