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Effects of local application of bovine amniotic fluid on fracture healing in rats (*Rattus norvegicus*)

Efectos de la aplicación local de líquido amniótico bovino en la curación de fracturas en ratas (*Rattus norvegicus*)

***Murat Tanrısever*[1]*, *Ozmen Istek*[2], *Hatice Eroksuz*[3], *Burak Karabulut*[3], *Erhan Cahit Ozcan*[4], *Muhammet Bahattin Bingul*[5], *Rıdvan Guler*[6], *Serkan Dundar*[7]**

[1]*Firat University, Faculty of Veterinary Medicine, Department of Surgery, Elazig, Turkiye.*
[2]*Mus Alparslan University, Faculty of Health Medicine, Department of Nursing, Mus, Turkiye.*
[3]*Firat University, Faculty of Veterinary Medicine, Department of Pathology, Elazig, Turkiye.*
[4]*Firat University, Faculty of Medicine, Department of Esthetic, Plastic and Reconstructive Surgery, Elazig, Turkiye.*
[5]*Harran University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Sanliurfa, Turkiye.*
[6]*Dicle University, Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Diyarbakir, Turkiye.*
[7]*Firat University, Faculty of Dentistry, Department of Peridontology, Elazig, Turkiye.*
**Corresponding Author: mtanrisever@firat.edu.tr*

ABSTRACT

In this study, it was aim to examine the local application of bovine amniotic fluid on bone fracture healing in rats. Twenty female sprague dawley rats included in the study were divided into 2 groups of 10. The sham group (n=10): Bone fractures were created in the right tibia bones of the rats and fixed with kirschner wire. After a four-week recovery period, the subjects were sacrificed. Local bovine amniotic fluid group (n=10): Bone fractures were created in the right tibia bones of the rats and local bovine amniotic fuid was applied during fixation with kirschner wire. After a four-week recovery period, the subjects were sacrificed. Samples from all subjects were decalcified, stained with hematoxylin and eosin, and new bone formation and fibrosis were analyzed. When the groups were evaluated in terms of new bone regeneration, it was determined that the new bone regeneration in the subjects treated with local bovine amniotic fluid were statistically significantly higher than sham group (P<0.05). When the groups were evaluated in terms of fibrosis, the fibrosis value in the sham group was found to be statistically significantly higher when compared with the local bovine amniotic fluid group (P<0.05). It can be stated that local bovine amniotic fluid application may positively affect the healing of bone fractures.

Key words: Bovine amniotic fluid; bone fracture; local application; bone fracture healing

RESUMEN

En este estudio, el objetivo fue examinar la aplicación local de líquido amniótico bovino en la curación de fracturas óseas en ratas. Veinte ratas hembra Sprague Dawley incluidas en el estudio se dividieron en 2 grupos de 10. El grupo simulado (n=10): se crearon fracturas óseas en la tibia derecha de las ratas y se fijaron con alambre de Kirschner. Después de un período de recuperación de cuatro semanas, los sujetos fueron sacrificados. Grupo de líquido amniótico bovino local (n = 10): se crearon fracturas óseas en los huesos de la tibia derecha de las ratas y se aplicó líquido amniótico bovino local durante la fijación con alambre de Kirschner. Después de un período de recuperación de cuatro semanas, los sujetos fueron sacrificados. Se descalcificaron muestras de todos los sujetos, se tiñeron con hematoxilina y eosina y se analizó la formación de hueso nuevo y la fibrosis. Cuando los grupos fueron evaluados en términos de regeneración ósea nueva, se determinó que la regeneración ósea nueva en los sujetos tratados con líquido amniótico bovino local fue estadísticamente significativamente mayor que en el grupo simulado (P<0,05). Cuando los grupos fueron evaluados en términos de fibrosis, se encontró que el valor de fibrosis en el grupo simulado era estadísticamente significativamente mayor en comparación con el grupo de líquido amniótico bovino local (P<0,05). Se puede afirmar que la aplicación local de líquido amniótico bovino puede afectar positivamente la curación de las fracturas óseas.

Palabras clave: Líquido amniótico bovino; fractura ósea; aplicación local; curación de fracturas óseas

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INTRODUCTION

Treatment of bone loss due to many diseases including osteoarthritis, osteogenesis imperfecta, osteoporosis, traumatic injury, cystic and tumoral lesions is difficult and requires a long process [1]. Delay in bone healing occurs with a high incidence and this is an important health problem [2]. About 5–10% of fractures undergo delayed healing or result in nonunion [3]. The most important step in the treatment of nonunion problems of broken bones is autologous bone grafting, in which bone fragments are taken from a secondary site and transferred to the fracture line in order to accelerate the healing process. Although autogenous bone grafting is accepted as the gold standard in bone healing, it can be faced with disadvantages such as morbidity in the donor area where the graft is taken and low quality autologous bone graft material [4, 5]. Researchers have been developing alternative treatment methods for delayed fracture healing for many years. Numerous approaches have been investigated to accelerate maturation of regenerated bone, such as growth factors [6], calcitonin [7], calcium sulfate [8], bisphosphonates [9], electronic [10] and ultrasonic [11] stimulation. Various factors are defined that support healing after fractures such as interleukins (IL); IL–1, IL–6, some growth factors; fibroblast, transformed and platelet–derived growth factors [12, 13].

Some researchers have explored the effects of human amniotic fluid, which is rich in growth factors, on fracture healing [13, 14, 15]. In addition, there is no literature on the effects of bovine amniotic fluid (BAF) on fracture healing. In the content of bovine amniotic fluid; mesenchymal cells, growth factors, macromolecules such as hyaluronic acid (HA) and hyaluronic acid activating agent have been reported [16, 17, 18, 19]. It is stated that HA suppresses the migration, proliferation and chemotaxis of lymphocytes, and also reduces scar formation by suppressing granulocyte phagocytosis and degranulation and macrophage motility [20].

The aim of this study was to investigate the effects of local use of bovine amniotic fluid which is thought to have a positive stimulating effect on fracture healing on fracture healing in a rat (*Rattus norvegicus*) tibia fracture model.

MATERIAL AND METHODS

This study was approved by Firat University (Protocol Number: 24/02/2020–380123) Local Animal Experiments Ethics Committee. It was carried out at the Firat University Experimental Research Center, and the Helsinki Declaration rules were strictly followed during the experiments. The rats used in the experiments were obtained from Firat University Experimental Research Center.

This study aimed to evaluate the effect of bovine amniotic fluid on fracture healing in the tibia of rats and histopathologically examine the bone tissues.

In this study, a total of 20 female Sprague–Dawley rats weighing 300–320 g were used. Rats were kept in 12 hours dark, 12 hours light environment and temperature throughout the study period. During the experiment, all animals were allowed easy access to feed and water.

The rats used in the experiment were randomly divided into two groups. In the sham group (n=10); rats tibias were cut with an electric bone saw and then repaired with kirschner wire intramedullary and no additional application was used. In the bovine amniotic fluid (BAF) (n=10) group; rats tibias were cut with an electric bone saw and then repaired with kirschner wire intramedullary and bovine amniotic fluid was applied to the fracture line.

BAF was taken from healthy pregnant cow by injector (ClickZip,Medical Device Manufacturer, Thailand) under aseptic conditions during cesarean delivery at Firat University Animal Hospital. The amniotic fluid was kept cold and transported to the laboratory. It was stored in a deep freezer (Arçelik, 2533D, Turkiye) at -20 °C until the day of surgery. It was used after waiting for 15 min to dissolve at room temperature.

Surgical procedures

The rats used in the experiments were administered intramuscular injection of 50 mg·kg^{-1} Xylazine hydrochloride and 5 mg·kg^{-1} dose of Ketamine hydrochloride for general anesthesia. All surgical operations on the subjects were performed in accordance with the required sterile surgical procedures. After general anesthesia, the tibial skin was shaved and cleaned. Before the incision, the knees were washed with 10% Povidone iodine. A linear incision of 1.5–2 cm in length was made on the skin of the tibia diaphyseal region. After passing the subcutaneous connective tissue, the tibiae were cut from the midline of the bone with an electric bone saw and divided into two parts. Then, the bones were reconnected with the retrograde intramedullary method with kirschner wires by using an orthopedic drill. While no extra agent was used in the sham group, 0.3 mL bovine amniotic fluid was applied to the surfaces of the fracture line in the BAF group. after the integration of the titanium implants the skins of the rats were then closed with suturing in their original position with polyglactin 4/0 suture material. As an antibiotic, all subjects recieved intramuscular 50 mg·kg^{-1} Cefazolin sodium for postoperative 3 days to prevent infection. As a pain reliever, 1 mg·kg^{-1} Tramadol hydrochloride was given intramuscularly for 3 days postoperatively. In this study, the recovery period was determined as four weeks, and at the end of this period, all rats were sacrificed.

Histopathological analysis

After the euthanasia procedure, the tibias removed as a whole were fixed in 10% buffered formalin solution for 3 days. The muscle tissues around the bones were then cleaned with the microtome (Leica RM2125, Wetzlar, Germany) blade and placed in a decalcification solution. Five days later, when the desired flexibility was achieved in the bone samples, the decalcification process was completed and the bone samples were placed in separate tissue tracking cassettes. After washing the samples under running tap water for about two hours, they were passed through alcohol, xylene and paraffin series in an automatic tissue tracking device and blocked with paraffin in a tissue blocking device. Serial sections 3–5 microns thick were taken from the paraffin blocks with a rotary microtome on positively charged slides. The prepared sections were stained with the hematoxylin–eosin staining method in an automatic tissue staining machine. The samples were semi–quantitatively evaluated in terms of fracture healing in the prepared preparations. The examinations were made with a trinocular light microscope (Olympus BX43, Tokyo, Japan) with a camera (Olympus DP72, Tokyo, Japan) and an imaging analysis system.

Fibrosis numbers were scored as followed: No fibrosis; 0, low-visible fibrosis; 1, mild–visible fibrosis; 2, and dense visible fibrosis; 3. Bone formation (BF) was scored as followed: No bone formation, 0; mild visible bone formation, 1; moderate visible bone formation, 2; and dense visible bone formation, 3 [21].

Statistical analysis

The SPSS 23.0 for Windows program (IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for statistical analysis. The data for each group was expressed as mean ± standart deviation. The Shapiro Wilks test was used to evaluate whether the data showed normal distribution. The data was found to be normally distributed and differences between groups were determined using the Student *t* Test. Significance was evaluated at the *P*<0.05 level (TABLE I).

TABLE I
Histologic New Regenerated Bone (NRB) and Fibrosis (FRB) parameters of the groups after the histological staining

Parameters	Groups	N	Mean	SD	*P**
NRB	Amnion	10	2,1	0,32	0,044
	Control	10	1,7	0,48	
FRB	Amnion	10	1,8	0,42	0,048
	Control	10	2,2	0,42	

SD: Standard deviation, *:Student *t* Test (*P*<0,05). Statistically significant difference detected between the groups

RESULTS AND DISCUSSION

No bacterial infection was detected in any of the rats included in the study during the experimental process. No fatal or nonfatal complications were detected during the experimental period. All rats succesfully completed the experiment. When the bone formation between the groups was examined, it was seen that bone formation was found to be statistically significantly higher in the group in which local bovine amniotic fluid was used compared to the results of the sham group (*P*<0.05)(TABLE 1). When the fibrosis values of the groups were examined, the fibrosis value of the sham group was detected statistically significantly highly than the group in which local bovine amniotic fluid was applied (*P*<0.05)(FIGS. 1 A,B,C,D and 2 A,B,C,D).

Amniotic fluid, contains cells from the amniotic membrane, decidual cells, decidual formations, and shed cells from the skin, digestive, respiratory, and excretory systems of the fetus. It consists of 99% water, inorganic and organic substances, salts and epithelial cells shed from the fetus. Half of the organic compounds are proteins and the other half are carbohydrates, fats, enzymes, hormones and pigments [22, 23].

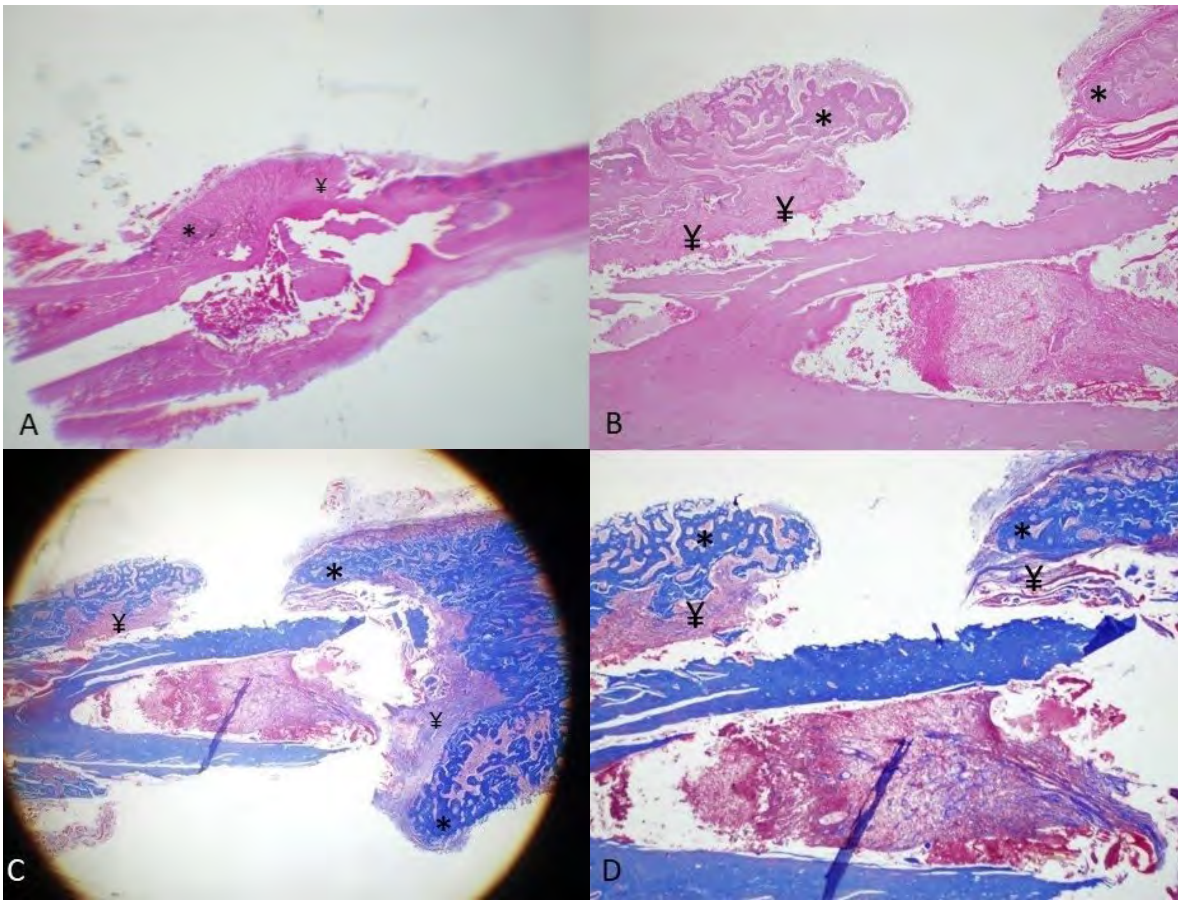

FIGURE 1. Decalcified histologic images of the *Sham Control Group*; (A:2X, B:10X magnification, Hematoxylin Eosine, C: 4X, D: 10X, Masson Trichrome). *: Newly Regenerated Bone, ¥: Fibrosis

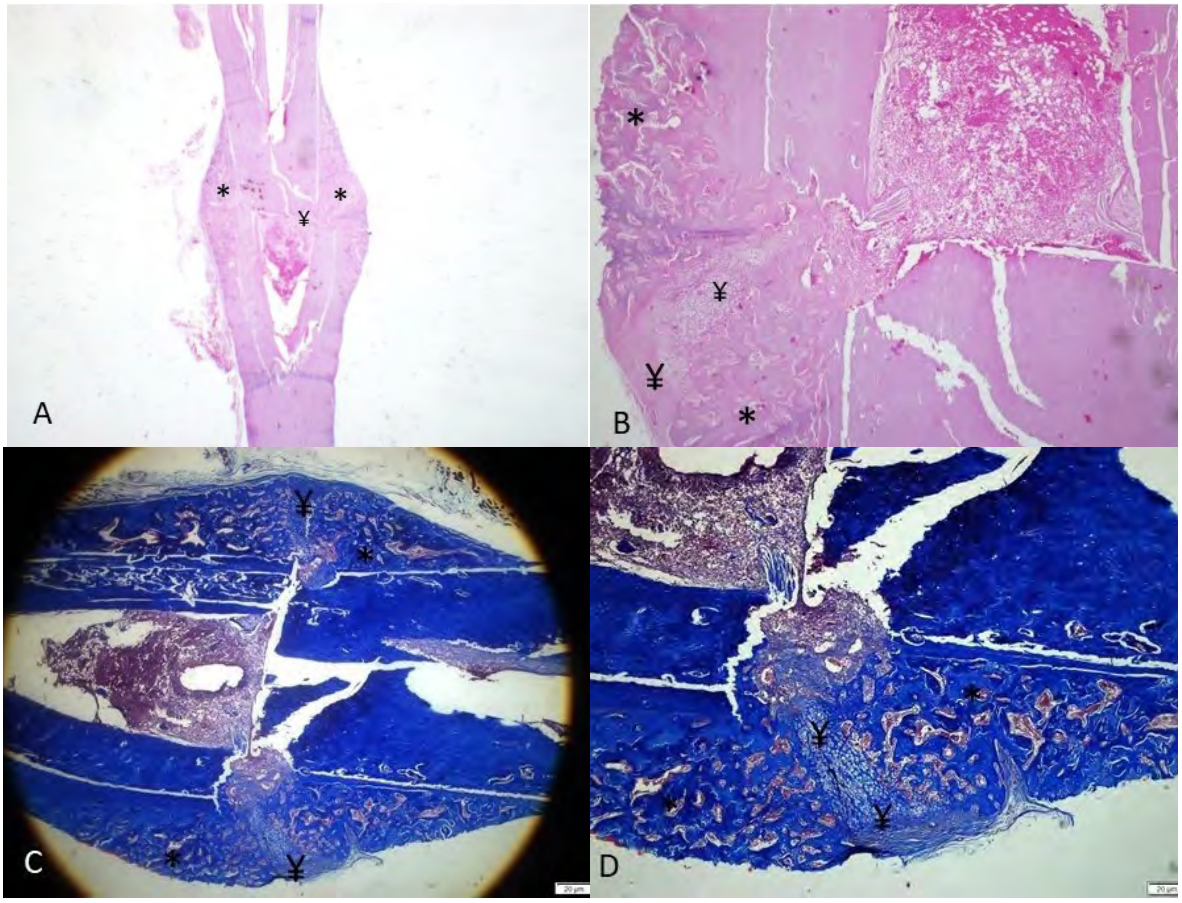

FIGURE 2. Decalcified histologic images of the *Local Bovine Amniotic Fluid Group*; (A:2X, B:10X magnification, Hematoxylin Eosine, C: 2X, D: 10X, Masson Trichrome). *: Newly Regenerated Bone, ¥: Fibrosis

Amniotic fluid accelerate cell epithelialization and prevent protein and fluid loss on the wound surface, thus reducing adhesion formation, while increasing fibroblastic activity with antibacterial and non–immunological power. Therefore, they contribute to collagen synthesis and reduce pain and inflammation through angiogenesis. In addition, amniotic fluid is a biological product that can be obtained easily and quickly, and its value is increasing [24, 25].

When the literature is examined, bovine amniotic fluid (BAF) contains macromolecules such as HA and HA activating agent, as well as insulin–like growth factor with activating on chondrocytes and mesenchymal cells, as well as other growth factors [19, 26, 27, 28, 29].

Karaçal *et al*. in an *in vivo* study, they reported that amniotic fluid can increase bone healing when applied to experimentally created rabbit (*Oryctolagus cuniculus*) calvarial defects by subperiosteal method [14]. According to the results of the study Karaçal *et al.*, it was reported that the local human amniotic fluid increased the bone healing in a statistically significant way compared to the sham group [14]. Due to bone regenerative capacity of bovine aminotic fluid, the present study aimed to investigate the effect of the usage of local bovine amniotic fluid on bone healing in experimentally created fractures of rat tibias. In the present study, when the new bone formation between the groups was examined, it was seen that new bone formation was found to be statistically significantly higher in the group in which local bovine amniotic fluid was used compared to the sham group (P<0.05). This shows that the present study is parallel with the literature.

Kerimoglu *et al*. showed in a study that human amniotic fluid had a positive effect on fracture healing in rat tibias [13]. In the study, it was stated that fibrosis was higher in the sham group compared to the other groups. In addition, Kerimoglu *et al*. evaluated bone healing with the scintigraphic method. It was suggested that human amniotic fluid may have positive effects on bone healing [13]. In another study, Gokce *et al*. reported that local human amniotic fluid increases bone healing in distraction osteogenesis studies in rabbit mandibles [15]. Researchers have reported that locally applied human amniotic fluid can improve new bone formation around the bone in maxillofacial operations such as distraction osteogenesis [15]. In the present study, when the fibrosis values of the groups were examined, the fibrosis value of the sham group was found to be significantly higher in the statistical evaluation when compared to the group in which local bovine amniotic fluid was applied. (P<0.05). Additonally new bone formation values in the experimental group; local bovine amniotic fluid group, detected higher compared with the sham group. The mechanisms of distraction osteogenesis and fracture healing are very similar. Considering this aspect, it can be stated that bovine amniotic fluid may have a similar effect on bone healing according to the results of the study carried out [15].

CONCLUSION

Within the limitaitons of this study, it is concluded that local application of bovine amniotic fluid may be effective in fracture healing. It is seen that there have not been any studies on fracture healing using bovine amniotic fluid in the literature before, and it can be thought that more detailed studies are necessary.

Conflict of interest

There is no conflict of interest.

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