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The Effect of Simultaneous Application of Blood Clot and Doxycycline on Bone Healing in Rabbit

Abstract

Background: Facilitating the fracture healing process is important to increase the fracture healing speed and to decrease the time period till union. This study aimed to evaluate the effects of blood clot and doxycycline on bone healing process.

Methods: Twenty mature male New Zealand white rabbits were used, in this study. A defect was created in the middle part of the radius. The rabbits were randomly distributed into 4 groups and the gaps were filled with the graft materials: blood clot, doxycycline, combination of blood clot and doxycycline and control group in which the defect was left empty. Radiographs of operated limbs were taken on 14th, 28th, 42nd and 56th postoperative days. Histologic samples were taken on the 56th day post surgery.

Results: On radiographic evaluation significant difference between the groups was not observed ($p>0.05$). On histopathological evaluation, blood clot and doxycycline groups were superior to control group ($P<0.05$), also combination of blood clot and doxycycline group was superior to other ones ($P<0.05$). There was no evidence of graft rejection in any group.

Conclusion: This study demonstrated that the combination of blood clot and doxycycline has a better function in bone healing process than other groups.

Keywords: Biomaterial, Blood clot, Doxycycline, Bone regeneration, Rabbit

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Introduction

Bone is a hard, specialized connective tissue with high resistance against pressure and damage [(1)]. This tissue is damaged due to trauma, developmental anomalies, oncologic resections, infection, delayed unions and non-unions, osteitis, osteotomies, bone cyst, and other factors [(2, 3)]. Auto graft, allograft, xenograft bone grafts, bone graft substitutes and recently nanomaterials and frameworks impregnated with various antibiotics can be used to accelerate the healing of these defects [(4)]. The desired bone graft has the characteristics of Osteoconduction, Osteogenesis and Osteoinduction. Auto graft is a combination of the above features, which requires a double surgery at the transplant site (usually the ileum junction) and imposes complications such as pain, increased possibility of infection, fractures and more blood loss to the patient. On the other hand, the amount of transplantable tissue that can be harvested is limited [(5)]. Allografts are more accessible than auto grafts, but their major disadvantage is the increased possibility of infectious diseases such as human immunodeficiency virus, hepatitis C, tuberculosis and rabies [(6-8)]. On the other hand, using xenografts increases the possibility of transmitting common diseases between humans and animals, such as swine flu virus, swine fever virus, and mad cow disease. Bone graft substitutes such as polymers, ceramics, and some metals are a way forward in cases of extensive skeletal deficiency, but they cannot regenerate bone tissue [(9)]. Doxycycline is a second-generation tetracycline antibiotic.

Recent studies have shown the anti-inflammatory, anti-catabolism and antioxidant effects of doxycycline and its bacteriostatic properties. The positive effects of doxycycline on osteoblasts and its negative effects on osteoclasts have been proven in laboratory and clinical cases [10-15]. The use of doxycycline may be able to prevent infection at the surgical site due to its bacteriostatic properties.

Bone healing occurs in two ways of primary healing (direct healing) and secondary healing (indirect healing or healing through callus formation). When the distance between two broken pieces of bone is less than 1 mm, the healing is of primary types, like bone stabilization with plaque. Secondary healing occurs when the distance between the two broken pieces of bone is more than 1 mm. When a fracture occurs, the local blood vessels are torn, bleeding occurs, and a hematoma is formed. Then the hematoma turns into fibrotic tissue, and in the next step, the fibrotic tissue turns into cartilage. Finally, cartilage tissue is calcified and turns into bone [16]. Both types of bone healing create a blood clot as a fibrin scaffold immediately after a bone fracture. In addition to having fibrin, the blood clot contains a significant number of platelets, erythrocytes, leukocytes, growth factors and signals, and minerals and nutrients, which affect the process of bone healing and regeneration [17].

An example of effective platelet compounds in bone healing is the Lyophilized Platelet, first used by Hafar et al. (2019) to heal long bones in the rabbit animal model [3].

Growth factors and signals such as vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGFβ1), and bone morphogenetic protein (BMPs) can be mentioned regardless of the presence of platelets in the blood clot from other components in the blood clot [17, 18].

Autologous blood clot, like auto graft bone tissue, is taken from the individual, which does not stimulate the immune system and has an effective role in accelerating bone healing. On the other hand, creating an autologous blood clot is a simpler process than obtaining an auto graft bone graft and does not have the mentioned disadvantages [19]. Gomes et al. (2003) proved the positive reparative role of doxycycline in jaw alveolar bone healing through increasing osteoblasts, decreasing osteoclasts and increasing Wnt activity [15], not related to its regenerative role in the experimental fracture of long bones. Therefore, this study was designed to experimentally evaluate the role of its restoration in long bones. This study evaluated the effects of autologous blood clots and doxycycline separately and combined on bone defect healing in the rabbit animal model.

Methods

This research was conducted in the surgery and radiology department of the Faculty of Veterinary Medicine of Shahrekord University, Iran, in 2019 (spring and summer). This study was approved by the ethics committee of the university. During the research period, all the rights of animals were respected under the principles of working with animals approved by the research council of faculty no. 170/1393.

Animal example

In this study, 20 adult male New Zealand white rabbits weighing 1600-2200g ages 12 months old were kept in the animal house for 15 days to adapt to the new conditions (light, food type, and storage). Rabbits received a subcutaneous dose of Ivermectin on day one and day 14. Doxycycline 50% was purchased in 100g powder packages from a drug store.

Preparation of blood clot and combination of doxycycline and blood clot

First, the syringe plunger was removed, and doxycycline powder was poured into the syringe chamber. Then, blood was taken with the same syringe from the rabbit heart that was anesthetized with ketamine, midazolam, and acepromazine. Antibiotic-clotted blood was stored for injection in the bone defect. Blood without antibiotics was also prepared and stored in the same way. The number of rabbits was noted on the related syringe for the bone defect of the same rabbit. Rabbits

were randomly divided into four groups of five, including control, blood clot, doxycycline, and combined blood clot and doxycycline groups.

Anesthesia

First, anesthesia was induced by intramuscular injection of a drug cocktail containing ketamine, midazolam, and acepromazine at doses of 30, 4, and 0.2 mg/kg, respectively. Then, anesthesia was continued until the end of the surgery by connecting the animal to an inhalation anesthesia machine containing isoflurane and oxygen through a mask.

Surgical procedure

The skin was cut on the anteromedial surface of the ulna after anesthesia induction, and a piece of bone about 10 mm was removed to create a bone defect. Then, the defect was filled with a doxycycline blood clot or blood clot only according to the above groupings. The rabbits were released freely in the cage without external fixation after recovery. Then, the rabbits were injected intramuscularly with the antibiotic enrofloxacin 10% at a dose of 10 mg/kg daily for three days. Rabbits were evaluated at clinical (daily), radiological (every two weeks), and histopathological (once at the end of the course) levels. The state of inflammation, infection, bleeding, and weighing in the target organ were considered in the clinical evaluation.

Radiological evaluation

The rabbits were mildly sedated at the end of weeks 2, 4, 6, and 8 after surgery and radiographed from the lateral-medial view of the right hand. Sandhu and Lane's modified system was used to evaluate and grade healing in radiology graphs Evidence of bone formation in the position is given a score of 4, and the fusion of this newly formed bone with the bone of the upper and lower part of the position is awarded a score of 2 separately. When the process of bone rearrangement is seen, two more points are added to the total of the above points. In the best case of bone healing, the total of these points reaches 10. The radiographs were evaluated by one person without knowledge of the studied groups, and all radiographs were evaluated only once [3, 20, 21].

Histopathological evaluation

The rabbits were anesthetized with a combination of ketamine and acepromazine on the 56th day after the surgery and euthanized by intracardiac injection of a high dose of magnesium sulfate. Then, the muscles and fascia were completely separated from the bone, and the radius and ulna bones were placed in 10% formalin and sent to the histopathology laboratory. The lamellae were evaluated histopathologically by light microscopy and four times magnification with the Emery grading system (Table 1) [21].

Radiographic and histopathological data were evaluated with Nonparametric Kruskal-Wallis's test and compared with Mann-Whitney U Test when $P<0.05$ and considered significant. SPSS software version 24 was used for statistical analysis.

Results

According to clinical observations, there was no infection or wound problem apart from reluctance to move in the first days.

Radiological results

The radiographic images of the surgical site in the control groups, blood clot, doxycycline, and the combination of the blood clot and doxycycline, were interpreted on days 14, 28, 42, and 56 after surgery. No significant difference was observed between the groups ($P>0.05$) (Table 2 & Figure 1).

The nonparametric Kruskal-Wallis's test was performed, and the difference between groups in different weeks was considered significant for $P<0.05$ to conduct the supplementary Mann-Whitney U Test.

Histopathological results

A significant difference was observed between the different groups in the histopathology evaluations on the 56th day after surgery ($P<0.05$). A significant difference was observed between the control group and doxycycline ($P=0.04$, $P=0.04$ and $P=0.01$, respectively). All treatment groups performed better than the control group (Table 3).

The combined blood clot and doxycycline group showed a significant efficiency ($P=0.01$ and $P=0.01$, respectively) compared to the

blood clot group and the doxycycline group (Table 3). No significant difference was observed between the blood clot and doxycycline groups (P>0.05) (Table 3). The fibrotic plaques in the control group prevented complete bone formation (Figure 2-a). The trabecular bone tissue can be seen well in the blood clot and doxycycline groups (Figure 2-b and Figure 2-d).

The formation of the bone marrow canal along with the trabecular bone tissue, which is turning into lamellar bone tissue, can be observed in the blood clot and doxycycline combination group, which is the best group from the histopathological view (Figure 2-d). In this group, all the slides obtained the highest possible score of 7 (Table 3).

Table 1: Histopathology evaluation using the Emery system

Degree	Observations
0	No sign of bone formation
1	Observation of fibrotic connective tissue alone
2	Observing the combination of fibrotic tissue and fibro cartilage with the predominance of fibrotic tissue
3	Observing the combination of fibrosis and fibro cartilage tissue with the predominance of fibro cartilage tissue
4	Observation of fibro cartilage tissue alone
5	Observing the combination of fibro cartilage tissue and bone with the predominance of fibro cartilage tissue
6	Observing the combination of fibro cartilage and bone tissue with the predominance of bone tissue
7	Observation of bone tissue alone

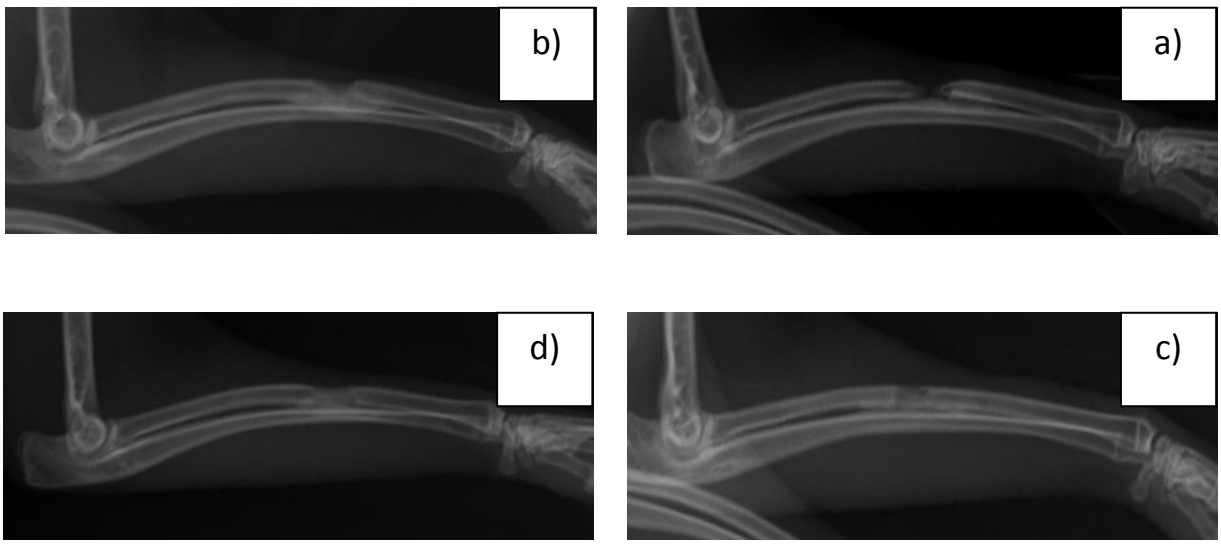

Figure 1: Radiographic images 8 weeks after surgery. a) Control group, b) blood clot group, c) doxycycline group, and d) blood clot and doxycycline combination group.

Table 2. Total radiographic scores of bone healing based on the Sandhu and Lane system

Group / Week	Control	Blood clot	Doxycycline	Blood clot and Doxycycline	P[a]
2	4(3-6)	2(1-3)	2(0-4)	1(0-3)	0.09
4	6(3-9)	6(4-7)	3(0-4)	5(3-8)	0.19
6	6(3-9)	7(6-9)	7(6-10)	7(5-9)	0.87
8	6(4-10)	7(7-10)	8(7-10)	9(9-10)	0.23

Table 3. Total histopathology scores of bone healing at week eight after surgery (Emery grading)

Group / Week	Control	Blood clot	Doxycycline	Blood clot and Doxycycline	P[a]
8	4(4-5)	[b]6(5-6)	[c]6(5-6)	[def]7(7-7)	0.009

a. The nonparametric Kruskal-Wallis's test was performed, and the difference between groups in different weeks was considered significant for P<0.05 to conduct the supplementary Mann-Whitney U Test.
b. A significant difference (0.04) was observed between the control group and the blood clot group, indicating the greater efficiency of the blood clot group.
c. A significant difference (0.04) was observed between the control group and the doxycycline group, indicating the greater efficiency of the doxycycline group.
d. A significant difference (0.01) was observed between the control group and the combination group of doxycycline and blood clot, indicating the greater efficiency of the combination group.
e. A significant difference (0.01) was observed between the blood clot group and the combination group of the blood clot and doxycycline, indicating the greater efficiency of the combination group.
f. A significant difference (0.01) was observed between the doxycycline group and the combination group of the blood clot and doxycycline, indicating the greater efficiency of the combination group.

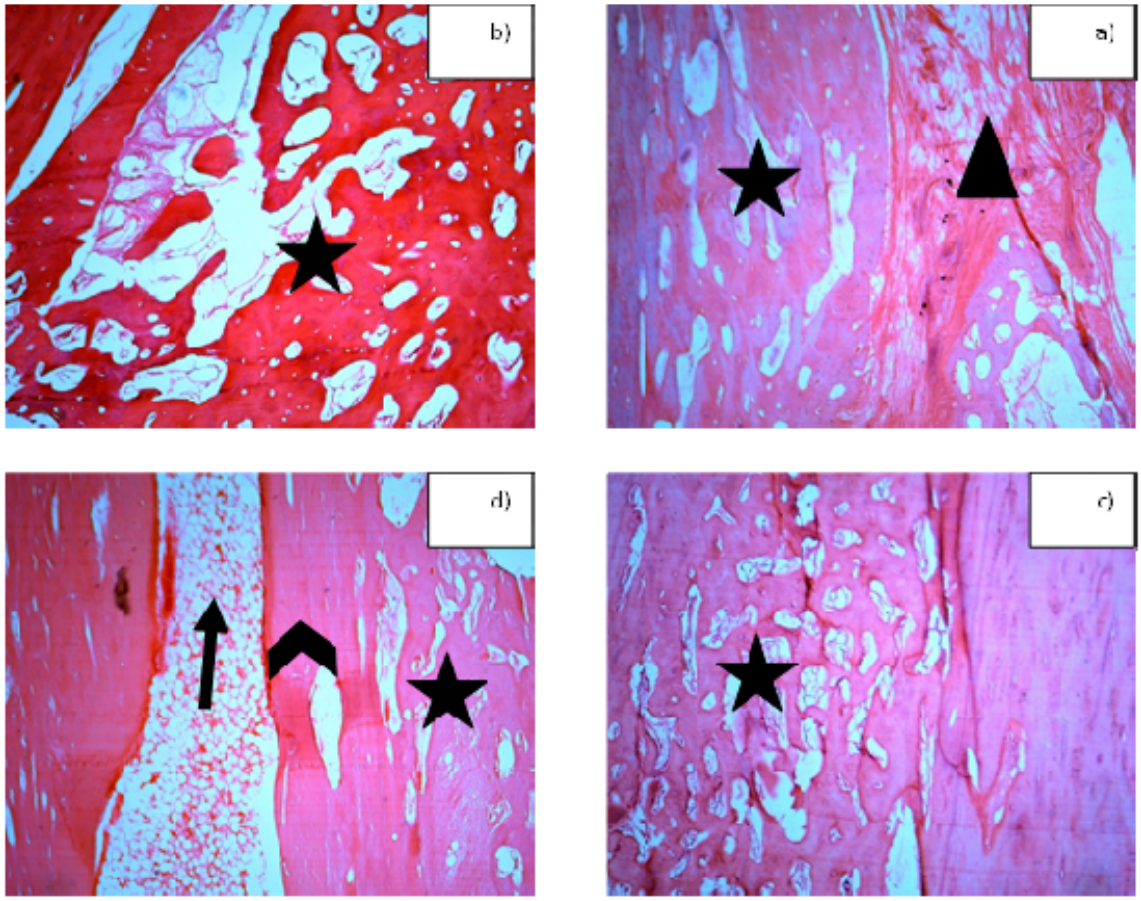

Figure 2: Histopathological sections from the control and transplant groups 56 days after surgery with 4x magnification. a) Control group, b) blood clot group, c) doxycycline group and d) blood clot and doxycycline combination group. Each of the symbols in these sections represents a specific texture:
▲ Fibrous plaque, ★ New trabecular bone, ^ New lamellar bone, and ⬆ New bone marrow channel

Discussion

This study used autologous blood clots as a fibrin scaffold to accelerate the early stages of bone healing. Doxycycline was applied as an inhibitor of destructive bone factors separately and in combination with each other to investigate their effect on bone defect healing in the rabbit animal model. Another research objective was to evaluate the effect of the autologous blood clot as connective tissue with bone formation, induction, and conduction properties, which do not stimulate the body's immune response [15, 22].

Tsunoda et al. (1993) separated cellular and extracellular elements of fracture hematoma and concluded that the cellular part of fracture hematoma could transform into chondroblast or osteoblast in laboratory conditions by adding ossification-inducing agents [22].

Grundnes et al. (1993) examined the rat femur and showed the significant and positive effect of fracture hematoma on fracture healing, while its removal causes a delay in bone healing. On the other hand, removing more hematoma by washing from the fracture site increases its destructive effect on bone healing [23].

Grundnes et al. (1993) studied the effect of hematoma on bone healing for four weeks in

rats and indicated no difference in the bone callus formation and the force required for fracture. However, bending moment and rigidity were more on the blood clot side [24].

Ozaki et al. (2000) found that fracture hematoma is necessary for proliferating bone periosteal cells [19].

Kolar et al. (2011) reported that the fracture hematoma adapts to hypoxic conditions, leading to increased angiogenesis, chemotaxis, and ossification. The increase of VEGF and IL6 stimulates vascularization, migrates leukocytes to the site, and increases osteoblastic biomarkers, such as SPP1, which enhances bone formation in hypoxia conditions [25].

Preininger et al. (2012) demonstrated that fracture hematoma is necessary for bone healing in the rat model, and adding bone morphogenetic proteins to the hematoma can completely heal the bone within six weeks [18].

Schell et al. (2012 and 2017) evaluated sheep tibial osteotomy and concluded that removal of the primary hematoma for increased surgeon visibility negatively affected bone healing, and secondary bleeding could delay healing and bone formation due to the lack of bone or cartilage-forming cells [26].

Previous studies have indicated that the main hematoma that forms immediately after the fracture should be preserved during the clinical treatment of fractures to benefit from its healing potential [27]. Platelets are also one of the compounds found in blood clots made by the fragmentation of megakaryocytic and have a great healing effect. In addition, platelets can regenerate bone tissue due to various growth factors such as platelet-rich, which was first used in bone healing by Schell et al. [27].

In this study, the blood clot group showed no significant difference from the diagnostic imaging point of view compared to other groups on days 14, 28, 42, and 56. However, the analysis of histopathological statistical data showed the superiority of this group compared to the control group. In this group, the trabecular bone tissue was well formed, and no fibrous tissue was observed in the position, unlike in the control group.

Another objective of this study was to assess the effect of the doxycycline antibiotic on bone healing.

Doxycycline is systemically and locally used to treat infectious diseases whole biological functions have also been proven completely independent of its antimicrobial properties. Golub et al. (1991) stated that this family of antibiotics inhibits the catabolism activities of human gelatinise and collagens enzymes in addition to their anti-inflammatory and antioxidant properties, which directly affect periodontitis and indirectly influence diseases such as osteoporosis, arthritis, and cancer [28].

Hanemaaijer et al. (1998) revealed that the synthesis of MMP-8 and MMP-9 decreases under the effect of therapeutic doses of doxycycline, which inhibits inflammation, and reduces angiogenesis and photogenesis in human endothelial cells [11]. Bezerra et al. (2002) proved the reduction of inflammatory bone resorption following the use of doxycycline [10]. Gomes et al. (2007) investigated the effect of adding two antibiotics from the family of tetracyclines, doxycycline, and minocycline, to the culture medium of human bone marrow osteoblastic cells in laboratory conditions and found a significant increase in osteoblastic cells and formation of the normal mineral matrix [13].

Zhang et al. (2007) showed that groups treated with doxycycline effectively inhibited mature osteoclasts and prevented osteoclastogenesis in laboratory conditions. According to this study, doxycycline can strongly inhibit the production of osteoclasts and osteolysis in clinical conditions [12]. Bedi et al. (2010) examined the effects of doxycycline on the inhibition of cell-matrix metalloproteinase and the healing process of muscles and tendons and observed the healing process by inhibiting MMP and increasing the organization of the collagen network [14]. Gunner et al. (2003) also obtained similar results [16].

In this study, the doxycycline group, like the blood clot group, did not show significantly differ from other groups in radiographic evaluation. However, the analysis of histopathological data showed the efficiency of this group. In addition, this group showed

the same function as the clot group, the trabecular bone tissue was well observed, and no fibrotic tissue was seen in the position.

The blood clot and doxycycline combination group did not show any significant difference in radiographic evaluation compared to other groups, but the analysis of histopathology data showed a significant efficiency of this group over other groups. The bone marrow canal was well formed, and the trabecular bone was turning into lamellar bone.

The amount of oxygen in the grafting material is effective in bone tissue healing. Therefore, scientists are recommended to assess the effect of blood clots on bone healing by taking blood from arteries and veins.

Conclusion

This study, like other studies, showed the positive role of the blood clot and doxycycline on bone healing and also revealed the increased efficiency of their combination.

Conflict of interest

The authors declared no conflict of interest in this study.

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