Concurrent Use of Theranekron with Hydroxyapatite on Bone Healing in Rabbit Model: Radiographic and Histologic Evaluation

Abstract
Background: Nowadays, bone grafting is used in both human and veterinary orthopedics to stimulate fracture healing, accelerate joint union, and restore bone defects. In such procedures, orthopedic surgeons are studying a favorable substitute for bone autograft. The present study aimed to evaluate theranekron and hydroxyapatite effects on bone healing in rabbit model.

Methods: First, 20 rabbits were prepared and divided into four groups of five. In theranecron group (T), theranecron was injected to the bone defect site on days 3, 7, and 10 after surgery, the other group was left empty as the control group, in the third group, a combination of theranekron and hydroxyapatite (T&H) was filled in the defect site, and in the fourth group hydroxyapatite was implanted alone (H). Radiographs were taken on days 14, 28, 42, 56 from rabbit’s hand after surgery in lateral view. At the end of the study, histopathological samples were taken from injured areas of radius.

Results: In radiographical and histopathological evaluations, T&H group had the best and the control group the weakest healing performance.

Conclusion: T and T&H groups were better than two other groups in terms of bone healing criteria, according to radiological and histopathological evaluation.

Keyword: Rabbit, Radius bone, Bone healing, Theranekron, Hydroxyapatite

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Introduction
Today, in human and veterinary orthopedics in vitro diagnostics, bone grafting is used to stimulate fracture healing, accelerate joint union, and repair bone defects. Autograft bone marrow are still considered as the gold standard for comparing other osteogenic stimuli. Autograft, in addition to stimulating healing, contains cells that do not stimulate immune responses and do not transmit contagious diseases\(^1\). In small livestocks, to collect autograft, ileal crest, the inner surface of the upper part of tibia, and the upper end of the humerous are used and in humans, ileal crest is used, too; however, this bone collection is associated with complications such as pain, infection, fracture, blood loss, and more surgical procedures, and the amount of bone taken is limited\(^1\). Currently, due to problems associated with bone autograft, the tendency to use bone allograft and xenograft has increased. First, there is no limitation on the amount of the graft taken, and it contains cells and protein substances stimulating bone healing. In addition, mechanicaally a supportive scaffold in large bone grafts, such as removal of tumors and loss of bone tissue\(^2\). However, in the use of allografts, there is a risk of transmission of contagious diseases. Synthetic hydroxyapatite, tricalcium phosphate and their combination are commonly used for bone grafting\(^3\). Hydroxyapatite has osteoconduction property and acts as a scaffold for the growth of osteoblasts without osteoconduction property\(^4\).
Tarantula cubensis is an extract widely used in treatment of tumors, abscesses, septicemia, and toxic diseases.\(^6\)\(^7\) It has also been claimed that in cow’s wound healing, it reduces inflammation and re-epithelialization on the 14\(^{th}\) day,\(^8\)\(^9\) and also contributes to the reduction of a broad range of infections.\(^10\)\(^11\)

Tarantula cubensis is from the mygalomorph species, called mygale cubensis, Tarantula cubensis, brown spider, and hairy spider.\(^12\)

This spider is native of South Carolina, Texas and Cuba. It has a dark brown color, a body filled with hair, and a toxicity of less than Tarentula Hispanica. The original composition of the entire body’s extract is extracted from the spider, with arachnid as the main base ingredient.\(^12\) The spider is first crushed and the alcoholic extract is extracted and sold commercially under the name of therankrone. Searching the resources and articles showed that therankrone has been used for tendon repair,\(^13\) but so far, its effects have not been studied on bone repair. The present study aimed to evaluate bone mineralization properties of combined hydroxyapatite (as bone graft conductor) and therankrone in healing rabbits’ bone defects.

**Methods**

The present study was conducted at Shahrekord University of Veterinary Sciences, Department of Surgery and Radiology in May 2017.

**Preparation of therankrone**

Therankrone (Darupakhsh Co., Iran) is available commercially as alcoholic soluble solutions at a dosage of 1 mg/kg.

**Animals used and grouping**

First, 20 pieces of native male rabbits weighing approximately 2 kg were purchased, Iormectin antiparasitic agent (Nasr Pharmaceuticals, Iran) was injected subcutaneously, and kept in fresh environment for 15 days and fed with standard rabbit food. Twenty rabbits were divided into four groups of five. In 5 rabbits, at the defect site, one mg/ml therankrone (T group) was diluted in 0.9% normal saline and injected at a dose of 1 μg/kg on days 3, 7, and 10 after surgery, and in 5 other rabbits as a blank control group (C group), the same volume were injected on the same days. Five other rabbits received hydroxyapatite (OS Satura®, Isotis Co., Netherlands) (H group), and in the other group, in addition to hydroxyapatite, therankrone was also injected in the same method described above (T&H group).

All stages of care and keeping animals during the study were according to the Animal Care Guideline published by the National Institutes of Health (NIH publication No. 85–23, revised 1985). The study was approved by the Ethics Committee of the Research Vice-Chancellor of Shahrekord University.\(^1\)

**The surgical procedure**

Rabbits were anaesthetized with 30 mg/kg ketamine and 0.2 mg/kg asecromazine by intramuscular injection, and their right hand was shaved and prepared for surgery. The skin was cut on the anterior–internal surface of radius bone and the radial bone was exposed by removing soft tissues and muscles. One bone section was removed twice its width (approximately 10 mm), and biomaterial was placed at the defect site by the method explained in the beginning of the material and methods. After the graft placement, the muscles were sutured and the skin was sutured subcutaneously with vycryl 00. After the rabbits recovered completely from anesthesia, they were released in cages without external stabilization. All rabbits received intramuscular injection of penicillin at a dose of 40,000 IU/day for 2 days once a day, and 12 mg/kg streptomycin.
Clinical evaluation
Rabbits were investigated and observed every day, and weight gain and hand use were recorded. Any local ulcers, inflammation, or lack of healing were considered.

Radiographic evaluation
Radiographs were obtained from the rabbits after the operation on the days 14, 28, 42, and 56 in lateral view. The film distance from the X-ray source was about 70 cm and the radiographic device was set at 45 kV (KV) and 20 mA/s. To evaluate and grade the radiographs, the modified Lane and Sandhu grading system was used, as follows (Table 1).

<table>
<thead>
<tr>
<th>Bone formation</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>No signs of bone formation</td>
<td>0</td>
</tr>
<tr>
<td>Bone formation and filling of 25% of the defect</td>
<td>1</td>
</tr>
<tr>
<td>Bone formation and filling of 50% of the defect</td>
<td>2</td>
</tr>
<tr>
<td>Bone formation and filling of 75% of the defect</td>
<td>3</td>
</tr>
<tr>
<td>Bone formation and filling of 100% of the defect</td>
<td>4</td>
</tr>
<tr>
<td>Upper and lower union</td>
<td></td>
</tr>
<tr>
<td>No union</td>
<td>0</td>
</tr>
<tr>
<td>Probable union</td>
<td>1</td>
</tr>
<tr>
<td>Complete union</td>
<td>2</td>
</tr>
<tr>
<td>Remodeling</td>
<td></td>
</tr>
<tr>
<td>No remodeling</td>
<td>0</td>
</tr>
<tr>
<td>Weak signs of remodeling</td>
<td>1</td>
</tr>
<tr>
<td>Complete remodeling</td>
<td>2</td>
</tr>
</tbody>
</table>

Histopathologic evaluation
The rabbits were kept for 8 weeks, sacrificed ethically, and sampled for histopathologic evaluation. In histopathologic evaluation, bone healing scoring method of Emery et al 1994 was used.

On day 56, all rabbits were sacrificed by intracardiac injection in an ethical manner after induction of anesthesia with magnesium sulfate. Then the radius bone was separated from each rabbit and the whole soft tissues around it were separated from the bone.

The bones were stored in 10% formalin, then mineralized in 10% organic citric acid for 4 days; after paraffinization and the remaining stages of preparation, they were checked with an optical microscope and graded according to Emery scoring system (Table 2).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty</td>
<td>0</td>
</tr>
<tr>
<td>Only fibrous tissue</td>
<td>1</td>
</tr>
<tr>
<td>Fibrous tissue more than fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td>Fibrocartilage tissue more than fibrous tissue</td>
<td>3</td>
</tr>
<tr>
<td>Fibrocartilage</td>
<td>4</td>
</tr>
<tr>
<td>Fibrocartilage tissue more than bone tissue</td>
<td>5</td>
</tr>
<tr>
<td>Bone tissue more than fibrocartilage</td>
<td>6</td>
</tr>
<tr>
<td>Bone tissue</td>
<td>7</td>
</tr>
</tbody>
</table>

Statistical analyses
First, the results were analyzed by Kruskal–Wallis non parametric ANOVA, when P-value<0.05, tested again by Mann–Whitney U test. In this test, P <0.05 were considered statistically significant. SPSS version 16 (SPSS, Inc., Chicago, USA) was used for statistical tests.

Results

Clinical evaluation
All rabbits had a very relaxed recovery from anesthesia after surgery. In the surgical site, there were swelling, inflammation, inability of weight bearing, and limping. Rabbits’ inflammation and swelling of the surgical site recovered until seventh day after surgery, and limping until the tenth day. No local and systemic infection was seen in any of the rabbits.

Radiographic evaluation
Radiographic evaluation showed healing process in rabbits in four groups of H, T, C, T&H groups on days 14, 28, 42, and 56 after surgery. The degree of bone formation, the amount of upper and lower union, and bone reformation (reconstruction) were evaluated (Figures 1, 2, and 3, Table 3).
Table 3. Comparison of radiographic evaluation between groups in different weeks; Median (minimum and maximum) (based on Lane and Sandhu)

<table>
<thead>
<tr>
<th>Group/week</th>
<th>T&amp;H group</th>
<th>H group</th>
<th>C group</th>
<th>T group</th>
</tr>
</thead>
<tbody>
<tr>
<td>2nd week</td>
<td>1(1–2)</td>
<td>2(0–2)</td>
<td>1(0–1)</td>
<td>1(1–2)</td>
</tr>
<tr>
<td>4th week</td>
<td>6(4–9)</td>
<td>5(3–9)</td>
<td>1(0–5)</td>
<td>5(2–9)</td>
</tr>
<tr>
<td>6th week</td>
<td>10(10–12)</td>
<td>5(9–10)</td>
<td>1(0–5)</td>
<td>9(8–13)</td>
</tr>
<tr>
<td>8th week</td>
<td>10(10–12)</td>
<td>8(4–10)</td>
<td>1(0–6)</td>
<td>8(1–8)</td>
</tr>
</tbody>
</table>

* Kruskal–Wallis non-parametric ANOVA was tested and in case of a significant difference between the groups (p value<0.05), Mann Withney U test was used.

b Significant difference was observed between T and C groups in the fourth (p = 0.04) and the sixth week (p = 0.008).

c In the sixth (p = 0.006) and the eighth week (p = 0.005), there was a significant difference between T and T&H groups.

d There was a significant difference between C and H groups in the fourth (p = 0.04), the sixth (p = 0.03), and the eighth week (p = 0.04).

e There was a significant difference between C and T&H groups in the fourth (p = 0.02), the sixth (p = 0.007), and the eighth week (p = 0.005).

f There was a significant difference between H and T&H groups in the sixth week (p = 0.03).

Figure 1: Radiographs of the second week: (a) H group, (b) T group, (c) T&H group, and (d) C group

Figure 2: Radiographs of the fourth week: (a) H group, (b) T group, (c) T&H group, and (d) C group

Figure 3: Radiographs of the eighth week: (a) H group, (b) T group, (c) T&H group, and (d) C group
The results of histopathologic examination of the samples
Radial bone was removed 56 days after operation, and the isolated samples were referred to the laboratory for histopathologic evaluation and bone healing grading was evaluated based on Emry system\(^{(1)}\). In histopathologic evaluation, no signs of inflammation or infection were detected in any of the rabbits. Statistical analyses of the histopathologic findings of the samples was investigated (Figure 4(a, b, c, d) Table 4).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>T&amp;H</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (0–3)(^{a})</td>
<td>13 (9–14)</td>
<td>13 (11–14)</td>
<td>5 (2–6)(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\)Kruskal–Wallis non parametric ANOVA
\(^{b}\)The control group had a significant difference with H and T&H groups
\(^{c}\)T group had a significant difference with H and T&H groups

The results of histopathologic evaluation was analyzed, like radiologic evaluation, with Kruskal–Wallis non parametric ANOVA test and compentary results using statistical test Mann–Withney U test. There was no significant difference between H and T&H groups. There was a significant difference between H and T groups \((p = 0.05)\), and between H and control groups \((P=0.05)\). There was a significant difference between T&H and T groups \((p = 0.03)\) and between T&H and C groups \((p = 0.03)\).

Discussion

Group H was significantly different from the blank control group and was better than this group. In a study in 2008, the important role of hydroxyapatite in accelerating bone repair has been confirmed, mentioned in this study for various reasons. Hydroxy enhances formation of new vessels at the defect site. It is suggested that the hydroxy zones allow the direct invasion of the newly formed vessels into minerals. Also, in cases with defects filled with hydroxyapatite, seeding and non–seeding angiogenic stages were observed. It also showed that H group caused formation of quasi–sinusoidal regions, which cause arterial thickness four folds the blank control group\(^{(16)}\).

The results of the study by Maimandi Parizi et al., in 2013, also confirmed the important role of hydroxyapatite in accelerating bone repair that approved the significant difference between the hydroxyapatite group and the control group, because calcium and phosphate are important bone mineral components and hydroxyapatite is the combination of these two. It also helps the physiological formation of the treated area, but its effect on intra–bone growth is still unclear. Also, in this study, there was a significant new bone formation in hydroxyapatite group than the negative control group. Also, other reasons of acceleration by hydroxyapatite in this study...
was that hydroxyapatite accelerates fracture repair by formation of hard and firm mechanical structure at the fracture site and conducting osteogenesis and angiogenesis. It has been shown that in case the fracture gap is large, hydroxyapatite has no effect on filling the defect, and it prevents bone tissue filling by forming fibrous tissue or fibrocartilage tissue. On the fracture edges, where the bone is directly in contact with hydroxyapatite, a new bone formation is observed in these areas that confirms the osteoconductive effect of hydroxyapatite, although this new bone formation on the edges is insufficient for filling the defect and union of the two sides of the fracture\(^{(4)}\).

In one study in 2010, hydroxyapatite proved to be able to provide phosphorus and calcium ions of the mineral environment in the bone that can accelerate bone metabolism, differentiate osteoblasts and synthesize collagen; also hydroxyapatite coating significantly accelerates bone defect repair stages and also improves the bioavailability level of carbon fiber\(^{(3)}\). The T group was significantly different from the control group. In this regard, and the impact of theranekron, various research has been done. The study of Adib–Hashemi et al. in 2015 indicates that the inflammatory cells of the group treated with theranekron were significantly lower than the control group and the collagen level was significantly higher. Histopathological observations have also pointed to an increase in granulomatous angiogenesis, and the repair process can accelerate by the decreased inflammatory cells that reduces local inflammation; thereby reduces the inflammatory phase and accelerates the recovery process. Also, the collagen levels were higher; given that collagen is used in the bone structure and repair process, this higher collagen levels as well as collagen accumulation and increased thickness accelerate the repair process. In histopathologic studies of T group, there was a significant improvement in angiogenesis, re–epithelialization and less inflammatory responses compared with the control group. Also, there was reduced inflammation and increased quality and rate of re–epithelialization in T group, compared with the control group. Collagen content was significantly higher in T group than in the control group; all of these properties could help accelerate the bone repair process\(^{(17)}\).

In the study by Oryan and associates, in 2014, radiographic images and histopathologic studies showed that the defect site in the control group and normal saline group was filled with fibrous tissue. That is why, when bone defect is filled with fibrous tissue, bone formation is not allowed to be filled with fibrous tissue, which is why in T group, because the fibrous tissue is less formed with respect to theranekron properties, the bone formation was faster in T group\(^{(18)}\). In the study by Oryan and associates, in 2012, on damaged tendons, it has been shown that theranekron reduces inflammation and, as a result, reduces inflammatory phase, and stimulates fibroplasia and scar tissue reformation during tendon repair; these criteria may improve the functional and structural properties of damaged tendons. Comparing the effects of theranekron on tendon repair, compared to control group, the overall biomechanical parameters improved in theranekron group compared to control group. The ultimate (stretching) strength of the damaged tendon treated with theranekron, compared to the healthy tendons of other organs regained 64% of their final strength and 164% improved strength with respect to the injured tendon of the control group, and these beneficial effects may be due to the effects of reduced inflammation by theranekron on inflammatory processes, as well as the effects of necrosis and fibroplasia after surgery. After tendon rupture, the inflammation process begins and the inflammatory cells migrate to the injury site. Cytokines and metalloproteinases, such as catepsin,
collagenase and elastase, are released by inflammatory and damaged cells. Therefore, most healthy collagen and matrix structures in the repair inflammatory phase are affected by inflammatory cells and matrix metalloproteinases. The event of these structural degradation changes by phagocytosis of the inflammatory cells and the lysing activity of matrix metalloproteinases reduces the biomechanical power of the damaged tendon site. The structural and biomechanical function of the damaged tendon, treated with theranekron significantly improved, compared with the control group, which may be due to the preservation of the principal (primary) collagens in the injury site. Protected fibers may act as a scaffold for tissue graft during subsequent fibromyalgia and initial rearrangement, similar to that used by synthetic fibrous implants used to repair the tendon. It may also play a role in collagen accumulation and conversion to thicker fibrils. Therefore, protecting the existing fibriles and increasing the growth of new fibrils, and thicker collagen fibriles indicate an accelerated maturation that may result in better performance and structure of theranekron–treated damaged tendons, compared to the control group. The effects of inflammation reduction of theranekron on inflammation will protect the damaged tissue from further destruction and increase tissue maturity. Theranekron group had better effects on weight bearing, mobility, and reduced tissue swelling in all weeks. This improvement can be attributed to decreased adhesion, edema, necrosis, limbing, and the effects of pain relief in theranekron.

May and colleagues believed in 1976 that theranekron would release maseration peptides, responsible for identification of fibrous tissue. In 2017, Ghasemi et al. concluded that theranekron significantly affects the activity of Caspise 3, which in turn fragments the intracellular DNA and ultimately stimulates cell death. Also, theranekron causes excessive increase in apoptosis and ultimately stimulates cell death. Naygul Stear et al. (2017) investigated the effects of theranekron on open wounds in rats and concluded that theranekron has significant effects on wound contraction and helps wound healing by forming a remarkable granulation tissue. The hydroxyapatite–theranekron group had a significant difference from the blank control group. Also, this group had a better function than all other groups, because, firstly, in the blank control group, the formation of fibrin tissue in the defect site prevented its filling by the bone. However, in hydroxyapatite–theranekron group, theranekron reduces the
production of fibrous tissue, the inflammation, and production of inflammatory cells, increases granulomatous angiogenesis, collagen accumulation, collagen thickness, and accelerates bone repair. Demarkive and necrotizing effects of theranekron can separate dead from living tissue and increase differentiation at the cellular level. May and colleagues in 1976 believed in 1976 that theranekron triggered the release of peptides responsible for specifying fibrous tissue. Hydroxyapatite also increases vascularization and vascular thickness and the presence of calcium and phosphorus in its structure, as a scaffold at the defect site of osteogenesis, significantly accelerates bone healing. Bigham and colleagues concluded in one study that hydroxyapatite group and powder of growth plate had a better effect on bone defect repair, compared to hydroxyapatite and powder of growth plate alone. This study also confirms our study on better results by combination of hydroxyapatite with other bone repair accelerators. As a result, these accelerated bone repair features were introduced for theranekron and hydroxyapatite, because in this group we have used both, so we expect better acceleration than the groups using each alone, as we have both bone acceleration properties in one group. The blank control group had a weaker function than other groups, because we did not use any substance or combination of accelerated bone repair. In this group, due to the formation of fibrous tissue at the defect site, this fibrous tissue disrupted the ossification process.

In the present study, the groups of theranekron and theranekron/hydroxyapatite performed better bone repair than in the other two groups.

Conflict of Interest
All authors hereby acknowledge that there are no conflicts of interest in this study.

References


