The effects of phytoestrogens on fracture healing: experimental research in New Zealand white rabbits

Fitoöstrojenlerin kırık iyileşmesi üzerine etkileri: Yeni Zelanda tavşanlarında deneysel çalışma

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BACKGROUND

Phytoestrogens are plant-derived natural molecules having some bone forming and bone substituting effects. In the present study, the role of phytoestrogens on bone healing was investigated in a rabbit fracture model.

METHODS

Twenty-two New Zealand white rabbits with right tibia fracture were divided into two groups randomly. The plant derived extract of *Vitex agnus-castus L.* (*Verbenaceae*) prepared before the study was administered intramuscularly in group 1 and group 2 was chosen as control. Fracture healing was monitored in weekly basis with blood alkaline phosphatase level, radiographs of extremities and 99m-Tc MDP bone scintigraphy. The study was finished at the end of the 3rd week. The extremities including tibial fractures were collected for histological examination.

RESULTS

Radiographic evidence of fracture healing obtained on postoperative day seven was superior in group 1 than control group ($p<0.01$). The 99m-Tc MDP bone scintigraphy uptake ratios on postoperative seventh day showed higher uptake in group 1 than in group 2 ($p<0.05$). The differences of scintigraphic uptakes in fractured tibias calculated on postoperative seventh day and postoperative 14th in group 1 were higher than group 2 ($p=0.04$). The histopathologic evaluation performed after sacrification of all rabbits on postoperative 25th day showed no significant difference between both groups. No statistical difference was determined related to the other variables.

CONCLUSION

Flavonoids affected positively the early periods of fracture healing mechanism in New Zealand white rabbits. We suggest further studies with phytoestrogens to determine the effects of various dosages and administration ways.

Key Words: Experiment; flavonoids; fracture healing; scintigraphy.

AMAÇ

Fitoöstrojenler bitkisel kaynaklı doğal moleküller olup kemik yapımını artırıcı ve kemik yerine kullanılabilme özellikleri vardır. Bu çalışmada, fitoöstrojenlerin tavşan kırık modelinde kırık iyileşmesi üzerine etkileri deneysel olarak araştırıldı.

GEREÇ VE YÖNTEM

Yirmi iki adet Yeni Zelanda beyaz tavşanı sağ tibia kırığı oluşturulduktan sonra randomize olarak iki gruba ayrıldı. Birinci gruba *Vitex agnus-castus L.*'den (*Verbenaceae*) çalışma öncesinde hazırlanan bitki ekstresi intramuskuler olarak enjekte edildi. İkinci grup kontrol grubu olarak seçildi. Kırık iyileşmesi haftalık olarak kan alkalen fosfataz düzeyi, direkt radyografi ve 99m-Tc MDP kemik sintigrafisi ile değerlendirildi. Üçüncü haftanın sonunda çalışma sonlandırıldı. Çalışma sonunda histopatolojik inceleme yapıldı.

BULGULAR

Yedinci günde yapılan direkt radyografik incelemede kırık iyileşme bulguları grup 1'de kontrol grubuna göre daha yüksek olarak bulundu ($p<0,01$). Yedinci günde yapılan 99m-Tc MDP kemik sintigrafisinde grup 1'de tutulum oranları kontrol grubuna göre daha fazla idi ($p<0,05$). Grup 1'de 14. ve 7. gün 99m-Tc MDP tutulum miktarları arasındaki fark kontrol grubuna göre daha fazla bulundu ($p=0,04$). Yirmi beşinci günde yapılan histopatolojik incelemede gruplar arasında anlamlı farklılık yoktu. Diğer değişkenler arasında farklılık saptanmadı.

SONUÇ

Flavonoid enjeksiyonlarının Yeni Zelanda beyaz tavşanlarında kırık iyileşmesinin erken döneminde faydalı olduğu gösterildi. Farklı doz uygulamaları ve veriliş yöntemlerinin farklı deneysel çalışmalarla araştırılmasını önermekteyiz.

Anahtar Sözcükler: Deney; flavonoidler; kırık iyileşmesi; sintigrafi.

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Vitex agnus-castus L. (*Verbenaceae*) is a shrub widely distributed in Middle East and Southern Europe. The fruits, flowers and leaves of *Vitex agnus-castus* contain high amounts of flavonoids, tannins, iridoids and diterpenoids.[1-3] There is a growing evidence that those plant derived flavonoids are protective against heart disease, by lowering serum cholesterol and dilating blood vessels, are cytotoxic and apoptotic against some cancer cells and useful in the treatment of some postmenopausal symptoms.[4-7] They have been shown to reduce bone loss and bone resorption in experimental models and in some clinical trials dealing with osteoporosis.[4,8-11] Various mechanisms of action of phytoestrogens have been proposed.[12-15] Genistein which is the major phytoestrogen of soybeans and soybean products has been shown to decrease the number of osteoclasts derived from rat bone marrow *in vitro*.[16,17] Effect of genistein on commitment and differentiation of bone marrow stromal cells to the osteoblast lineage has been reported. But, they do not influence the late osteogenic maturation markers.[18] Daidzein and genistein have been found to have stimulatory effect on protein synthesis and on alkaline phosphatase release by various types of osteoblast cells *in vitro* and genistein has been shown to prevent bone resorption via a paracrine mechanism.[14] Recently, several researchers have reported their bone preserving, bone substituting and bone augmentation effects when used locally in experimental studies.[19-21] However, the effects of flavonoids on fracture healing still remain undocumented and there are limited number of studies dealing only with bone defects and supplemental bone graft materials.[19,21,22]

This study focuses on the effects of phytoestrogens on fracture healing in an experimental rabbit model.

MATERIALS AND METHODS

The fruits of *Vitex agnus-castus L.* were collected in July 2005. A voucher specimen (no. 1262) is deposited in the Herbarium of the Faculty of Pharmacy, Department of Pharmacognosy. The fruits of the plant were dried at room temperature and then reduced to coarse powder. Twenty g of the sample was extracted with ethanol at room temperature, under stirring for 2 days. The extraction solvent was evaporated to dryness in vacuo. Ethanol extract was referred to as vitex extract (VE), and its yield was 7.26%. Sample solution was prepared by dissolving VE (5.2076 g) in 10 ml of 0.9% saline solution with an ultrasound mixer in biochemistry laboratory. It was then filtered through a membranous filter with a pore size of 0.45 µm. The extract was ready for intramuscular (IM) injection to the rabbits.

Twenty-two mature New Zealand white rabbits weighing 1.62±0.05 kg were picked up and kept in a wire top cage with free access to tap water. They were administered with standard food. For the anaesthesia procedure first sedation was performed by IM injection of 3-5 mg/kg Alfazin® (Ege Vet). Following to this, general anaesthesia was performed by intravenous (IV) injection of 7.5-15 mg/kg Propofol® (Abbott). Right tibia fracture was created under general anaesthesia with a three-point bending moment. The fractured tibias were then fixated with a 1.5 Kirschner (K) wire intramedullary in a closed fashion through a stab incision at the knee joint proximal tibia. The upper ends of K-wires were buried under the skin. After the operation, the operated legs were splinted. They were randomly assigned to two groups after the operation. The rabbits in group 1 were injected with 0.75 mg of fruit extract im (group 1). The other group was left as a control group (group 2). The same amount of im injection was repeated every other day. Each rabbit in group 1 was totally injected for five times.

The X-rays of the rabbits from both groups were taken just after the operation, on the 7th, 14th and 20th day after the operation. The fracture healing was evaluated according to Lane and Sandhu[23] criteria. This criteria system includes bone formation, ossification of the fracture site and remodelling of the fractured bone (Table 1). The fracture healing was also assessed with 99m-Tc MDP three phase bone scintigraphy on 7th, 14th and 20th day after the operation. The scintigraphic uptake was measured and compared with the opposite extremity. The results from bone scans were analyzed using the computer to measure the radioactivity within regions of interest. In calculation of the uptake, we assigned a 1 cm^2 area which was in accordance with the fracture site in order to compare the amount of uptake to the opposite tibial region. The values were then divided and the calculated ratio was used for statistical analysis. The value of 99m-Tc MDP three phase bone scintigraphy on fracture healing has been established.[24,25]

The fracture healing was evaluated histologically in four periods in rabbits:

1. Hematoma: First 5 days,
2. Chondrogenesis: 5-14 days,
3. Enchondral ossification: 14-21 days,
4. Remodelling period: After 21 days.

Following the sacrification of all rabbits on the 25th day after the operation the operated extremities were collected and sent for microscopic examination. The tibias containing fracture regions were fixated with 10% formaldehyde solution for 3 days. For decalcification, they were embedded in 10% phormic acid solution for 15 days. The sagittal and axial plane samples were drawn from fracture healing sites. After dehydration, they were buried in paraphine blocks. The 4 mm samples were prepared and stained with haematoxylen and eosin. The fracture healing was evaluated with the criteria of Heiple et al.[26] This histological criteria consisted of ossification, assessment of spongious bone, cortical region and bone marrow (Table 2).

The serum alkaline phosphatase (ALP) levels were measured and documented on 7th, 14th and 20th day after the operation. The measurements were analyzed in biochemistry laboratory.

The study design was approved by the Local Ethics Committee for animals prior to performing the study.

Statistical analysis was performed using SPSS 10.0 version for Windows program (SPSS Inc., Chicago, IL, USA). In statistical analysis Mann-Whitney U-test, Wilcoxon signed ranks test, and Kolmogorov-Smirnov test were used for evaluation. Statistical significance was assigned to p values less than or equal to 0.05.

RESULTS

Totally 4 rabbits, 2 rabbits from each group, were dead before the completion of the experiment. Two groups did not show difference regarding the dead rabbits.

The mean X-Ray scores for the evaluation according to the Lane and Sandhu[23] criteria on the 7th postoperative day for group 1 and group 2 were 4±2.4 (median: 3.0, 25%: 2.0, 75%: 6.0) and 1.3±1.89 (median: 1.0, 25%: 0, 75%: 1.5), respecti-

Table 1. Radiographic scoring system

Category	Points
Bone formation	
No bone formation	0
Bone formation equal to 25%	1
Bone formation equal to 50%	2
Bone formation equal to 75%	3
Bone formation filling the fracture site	4
Union	
Non-union	0
Possible union	2
Radiographic union	4
Remodelling	
No remodelling	0
Remodelling of intramedullary canal	2
Complete cortical remodelling	4
Maximum score	12

Table 2. Histologic scoring system

Category	Points
Union	
No sign of union	0
Fibrous union	1
Osteochondral union	2
Bone union	3
Complete reorganization of bone	4
Spongiosa	
No cellular activity in bone	0
Early bone healing	1
Active new bone formation	2
Reorganised spongiosa formation	3
Completely reorganized spongiosa	4
Marrow	
None	0
Replaced by fibrinous material	1
Marrow more than 50% replaced by new tissue	2
Marrow completely replaced by new tissue	3
Adult-type fatty marrow	4
Cortex	
None	0
Beginning to appear	1
Formation well under way	2
Completely reformed	3
Complete reorganisation	4
Maximum score	16

vely. These scores showed significant difference ($p<0.01$, Mann-Whitney U-test) (Fig. 1, 2). The evaluation for the other X-Rays between groups yielded no significant difference.

The mean ratios of 99m-Tc MDP three phase bone scintigraphy on the 7th postoperative day for group 1 and group 2 were 4.18±1.10 (median: 4.38, 25%: 3.19, 75%: 5.16) and 3.07±3.25 (median: 1.72, 25%: 0.93, 75%: 4.41), respectively. These measurements yielded significant difference ($p<0.05$, Mann-Whitney U-test) (Fig. 3). The difference in percentage between scintigraphic uptake measurements of operated right tibias calculated on 7th and 14th postoperative day for group 1 and group 2 were 124.99±223.06 (median: 50.43, 25%: -10.30, 75%: 162. 89) and 52.09±69.64 (median: 42.05, 25%: -2.11, 75%: 96.65), respectively. These measurements yielded significant difference ($p=0.04$, Wilcoxon signed ranks test) (Fig. 4). No significant difference was detected for the rest of the comparisons.

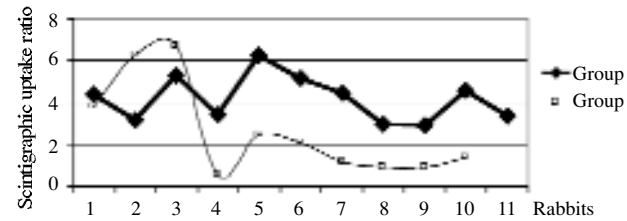

Fig. 3. The scintigraphic uptake ratios on 7th day measurements.

Macroscopically, we saw no abnormal motion at the fracture sites. In the histological evaluation performed at the end of study, no significant difference was detected between both groups according to criteria of Heiple et al.[26] Well-shaped mature bony trabeculi that were longitudinal according to the fracture site were surrounded with osteoblasts intramedullary in all of the samples except 2 (1 from each group) in both groups. Repair tissue was rich in vascular structures. Intramembraneous ossification was superior to endochondral ossification. Inter-

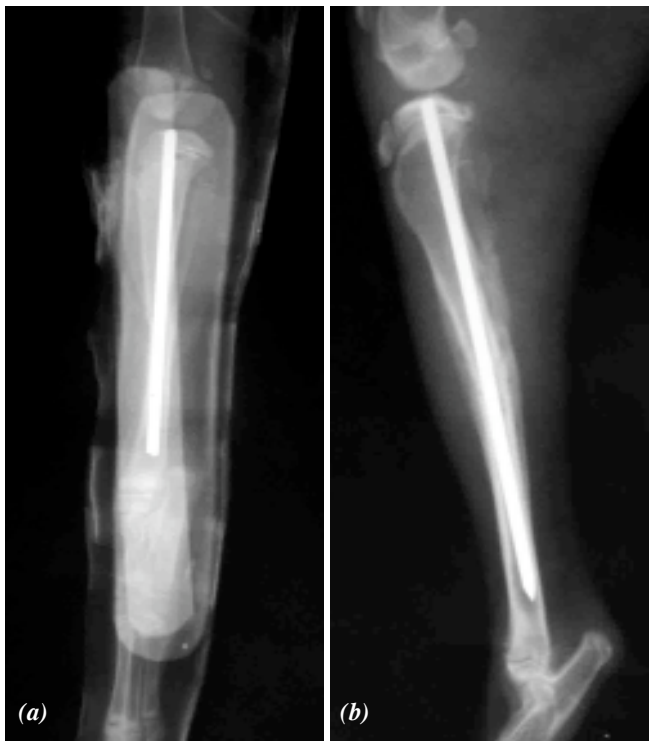

Fig. 1. Radiographs of rabbit from group 1 is shown. **(a)** Early postoperative radiography shows the fracture fixated with Kirschner wire. **(b)** Postoperative 7th day radiography shows radiographic callus formation filling the fracture site and possible union.

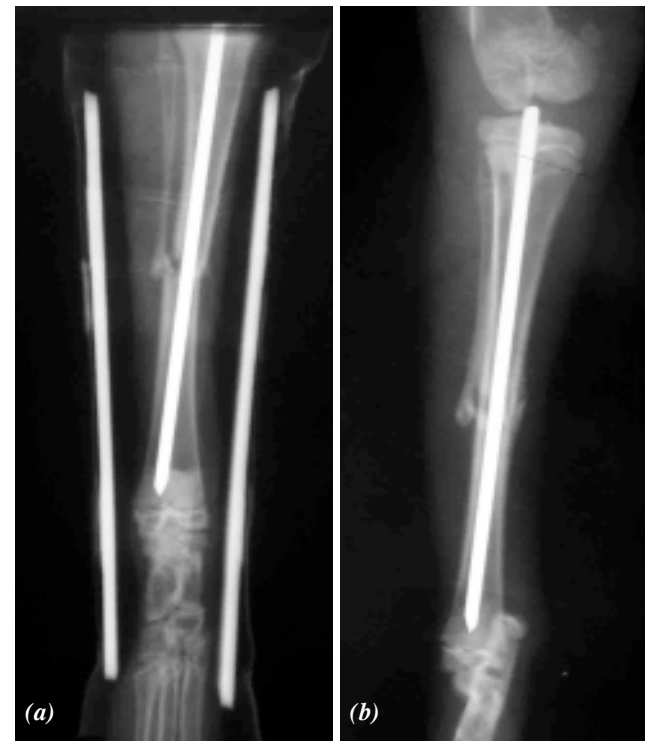

Fig. 2. Radiographs of rabbit from group 2 is shown. **(a)** Early postoperative radiography shows the fracture fixated with Kirschner wire. **(b)** Postoperative 7th day radiography shows radiographic callus formation filling the 50% of the fracture site.

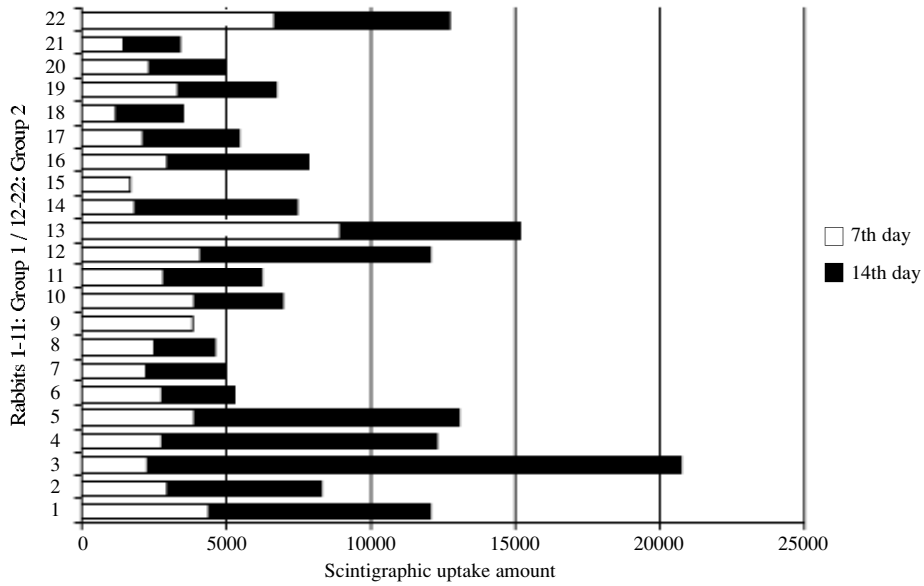

Fig. 4. The scintigraphic uptake amounts on 7th and 14th days measurements.

trabecular distance was narrower and lamellar structure was seen through cortical region.

The biochemical evaluation for alkaline phosphatase showed no significant difference for all comparisons.

DISCUSSION

In the current study, it was shown that phytoestrogen injections provided acceleration at early phases of fracture healing since the scintigraphic uptake and direct radiological examination yielded significant difference on 7th day but the evaluation on 14th and 20th day did not show any significant change. In addition to that, scintigraphic uptake also yielded significant difference between measurements performed on 7th and 14th day postoperatively. The process of bone formation follows an orderly cascade of events including initial inflammatory phase characterized by an increase in regional blood flow, invasion of neutrophils and monocytes, removal of cell debris and degradation of the local fibrin clot, revascularization phase associated with the invasion of new blood vessels formed by endothelial progenitor cells and subsequently a cell proliferation phase during which there is multiplication of a highly proliferative population of connective tissue progenitor cells and remodeling phase being the final phase of bone formation characterized by the systemic removal of the initial matrix and tissues that formed in the site primarily through osteoclastic and chondroclastic resorption and their replacement with more organized lamellar bone aligned in response to the local loading environment. Vascular relaxation with increase in regional blood flow, inhibition of macrophagic and osteoclastic activities and induction of formation of highly proliferative connective tissue progenitor cells might be some of the most likely mechanisms.[12,13,27] The vasodilatory effect of short-term phytoestrogen treatment on carotid and cerebral arteries in male rats has also been reported.[27] Nitric oxide mediated vasodilation has been shown to be present around a fracture site and maximal in the early healing phase, before returning to basal levels as healing progresses in an experimental study. This is compatible with an initial restoration of blood flow at a fracture site by nitric oxide dependent vasodilation of preexisting blood vessels, followed by ingrowth of less nitric oxide dependent angiogenic vessels during the later phase of repair.[28] It has been shown that isoflavone equol rapidly leaded to the activation of NOS and increased nitric oxide production.[12] Nitric oxide mediates the function of bone cells and remodelling of bone.[29,30] Baldik et al.[31] have shown in their experimental study that nitric oxide might be useful as a therapeutic adjuvant in clinical situations when local formation of bone was needed. Moreover, when combined appropriately, treatment with orthotopic nitric oxide supplementation and

systemic inducible nitric oxide synthase inhibition may enhance bone healing. One of the reasons why we found difference in early phases of bone healing might be concerned with the vasodilatory effect of phytoestrogens by induction of nitric oxide production. In addition to their vasodilatory effects, flavonoids also have been mentioned to alter the metabolic balance towards bone production by inhibiting the macrophagic and osteoclastic activities.[15,19] Yamagishi et al.[15] stated that genistein might have inhibited the formation of osteoclast-like cells. In their study with human primary bone marrow cells, Heim et al.[18] showed that phytoestrogens enhanced osteogenesis and repressed adipogenic differentiation. More recently, genistein has been shown to stimulate the production of osteoprotegerin, a member of the tumor necrosis factor receptor superfamily preventing bone resorption via a paracrine mechanism.[14] Osteoprotegerin provides a further mechanism for the bone-sparing effects of isoflavones. Yamaguchi and Ma[32] stated that genistein had a unique anabolic effect on bone calcification *in vitro*. We think that the alteration of metabolic balance towards osteogenesis might also be effective in having different results in the phytoestrogen injected group.

Phytoestrogens have recently been shown to be used as bone substitutes and supplemental bone graft materials.[19,21,22] Merolli et al.[19] designed an experimental study by using a soybean-based biomaterial that were filled a predrilled femoral cavity in New Zealand white rabbits and found the retrieved operated femurs had showed a macroscopic appearance similar to the nonoperated controls. In the same study, they concluded that the released genistein found in granules shifted metabolic balance towards bone production by inhibiting the macrophagic and osteoclastic activities and that the material degrading surface supported the apposition and mineralization of newly-formed bone. Naringin which is a flavonoid has been shown to increase new bone formation locally when used as a graft material and affect bone cell activities *in vitro* and at high concentrations significantly increased the activity of alkaline phosphatase up to 20% in experimental studies.[21,22] Similarly, in their experimental study, Minegishi et al.[20] found that ipriflavone, which is a flavonoid, affected the quality of bone augmentation at an early stage. Our result was consistent with the literature that the vitex extract containing flavonoids that we injected affected the fracture healing site in accelerating the fracture healing process in early phases.

Variable effects of flavonoids on ALP have been reported.[15,32,33] Yamaguchi and Ma[32] designed an *in vitro* study to determine the effect of polyphenols on calcium content and alkaline phosphatase activity in male rat femoral tissues and found that different concentrations of genistein affected the diaphyseal alkaline phosphatase levels in opposite directions. It may be thought that effect of genistein on diaphyseal alkaline phosphatase levels was dose-dependent. In another study, Yamagishi et al.[15] reported that genistein had no effect on the expression of ALP. Opposite to that, *in vitro* stimulatory effects of flavonoids on ALP release have been reported.[33] We found that serum ALP levels showed no significant difference during the experiment. We think that the reason why we did not find difference in ALP levels might be related to the dosage of plant extract we injected had no increasing effect on serum ALP activity.

As a conclusion, phytoestrogens affected the early periods of bone healing positively. Further experimental and clinical trials are needed to gain further understanding on its effects on fracture healing mechanism as well as different amounts of dosages.

Acknowledgements

We specially thank to Dr. Beytiye Baş with her kind help in making scintigraphic scans, measurements and evaluation.

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