Enhanced healing process of fracture by treatment of Kaempferol.

Lich Thi Nguyen[1#], Otgonbayar Maidar[1#], Ashish Ranjan Sharma[1#], Garima Sharma[1], Heung Cheol Kim[1], Seonghun Park[1,2], Sang-Soo Lee[1*], Ju-Suk Nam[1*]

[1]Institute For Skeletal Aging & Orthopedic Surgery, Hallym University-Chuncheon Sacred Heart Hospital, Chuncheon 24252, South Korea
[2]School of Mechanical Engineering, Pusan National University, Busan 46241, South Korea
[#]Authors contributed equally to this work.

Abstract

Recently, plant-derived phytoestrogens have been shown to possess promising results for bone preservation and bone augmentation. Kaempferol, one of flavanols, is known to possess osteogenic effects and ability to stimulate bone formation. This study was designed to investigate the effect of Kaempferol on the process of bone healing in standardized mouse tibia fracture model. Oral administration of Kaempferol (0.2, 0.5 and 5 mg/kg body weight) to mice with fractured tibiae demonstrated an increase in callus formation and new bone formation as evidenced by the measurement of callus diameter and H&E staining, respectively at 21 days post-fracture. In addition, radiographic examination carried out at early stages of fracture healing process (11 days and 14 days post-fracture) revealed prior appearances of denser and bigger calluses in Kaempferol (0.5 mg/kg) treated group than that from control. Taken together, it can be concluded that Kaempferol potentiates fracture healing by increasing callus formation and initiating bone remodeling process.

Introduction

Fracture healing is a physiological process that relates to bone regeneration. It is a dynamic process coordinated by multiple cell activities including cell proliferation, differentiation, migration, angiogenesis, and remodeling. Fracture healing is divided into four overlapping biological stages namely, the early inflammatory response, soft callus formation, hard callus formation and initial bony union followed by bone remodeling process. Early inflammatory stage occurs immediately after a fracture with hematoma formation and repair cells recruitment initiated by a cascade of growth factors and cytokines secretion. Secreted factors facilitate the recruitment of mesenchymal stem cells (MSCs) which then start differentiating into specialized cells to build new bone tissue (osteoblasts) and new cartilage (chondroblasts). The second stage is characterized by the formation of soft callus, carried out by chondrocytes and fibroblasts. Both of these cells build a semi-rigid soft callus for providing mechanical support to the fracture and at the same time act as a template for the bony callus. The third stage is of hard callus formation in which osteoblasts have a major role to play and are responsible for initial woven bone matrix. Henceforth, the stage is regarded as the primary bone formation event and represents the most active stage of osteogenesis. The final stage is a remodeling phase of action of osteoblast (bone forming cells) and osteoclast (bone resorption cells) to remodel callus into stronger lamellar bone [1] and is often termed as secondary bone formation. However, once in a while fracture healing may be delayed or impaired by some complex clinical conditions, including osteoporosis, resulting in tardy or permanent failure of healing (nonunion) as well as survival of injures. During such state, normal regenerative process is generally compromised and patients require quite a few months to achieve an adequate mechanical competence to support normal physiological loads.

Therefore, therapies focusing on improving quality and the rate of repair of fracture healing are required. The search of compounds that could efficiently improve repair process under less ideal conditions would be of significant importance for both social and economic benefits [2, 3, 4].

In the quest of above said objectives, several therapeutic agents have been intensively reported to improve fracture healing including estrogen hormone, growth factors (bone morphogenetic proteins, BMPs) and osteoporosis drugs (parathyroid hormone, bisphosphates) [2, 5, 6, 7].

However, there are some significant limitations of using current available agents. Use of estrogen therapy may increase risk of hormone-sensitive cancers while adverse effect of delayed remodeling process is being found associated with the treatment of bisphosphates [8], and there are several concerns on delivery system, bioactivity and limitation of the case treatment related to BMPs [9, 10]. Until now, no systematic agent has been registered with the implications of improvement of fracture healing. Moreover, considering the drawbacks associated with above mentioned therapies, new safe and bioactive agents are urgently required to manage bone fracture healing process with minimal side effects [11].

Owing to the absence of clinical side effects and cheaper alternatives to available therapies, traditional integrative medicine has also been considered on improving effectiveness of fracture healing both in animal models as well at clinical level [12, 13, 14]. Flavonoids, a type of natural compounds that are found in many kinds of fruits, vegetables, tea and traditional herb medicine have been reported to exert their effects on reducing bone loss and promoting bone formation. Recently, scientists observed positive effect of phytoestrogens and formononectin on early fracture healing [15, 16]. The flavonol Kaempferol is another popular flavonoid which is present in a variety of vegetables and fruits and has been reported to have protective effect against bone loss and increase bone strength both *in vitro* and *in vivo* models [17, 18, 19]. It is suggested that Kaempferol may be a promising agent for treatment of postmenopausal osteoporosis [20]. Kaempferol is known to possess anti-osteoclastogenic effects *in vitro* such as induction of osteoclast apoptosis and inhibition of osteoclastic bone resorption [21, 22]. Moreover, Kaempferol has been shown to promote osteoblast function *ex vivo*, induce bone formation *in vivo* [23] and protect osteoblast from damage by oxidative stress [24]. In addition, Kaempferol has been demonstrated to induce osteoblastic differentiation in rat primary osteoblasts [25], murine mesenchymal cell line and human primary mesenchymal stem cells [26]. Chondrogenic differentiation and formation of cartilage nodules can also be stimulated by treatment of Kaempferol in pre-chondrogenic, ATDC5 cell line [27].

However, there is no study available for the effect of Kaempferol on fracture healing process in the literature. Therefore, we aimed to evaluate the effect of Kaempferol on fracture healing process using murine tibial fracture model produced by standardized equipment.

Materials and Methods

Generation of Standardized Fracture of Mouse Tibia Shaft

Eight-week-old outbred ICR (Institute of Cancer Research) Swiss mice weighing 29.0 ~ 30.5 g were purchased from Taconic Inc., South Korea. All the experimental studies on animals were approved by the institutional ethical committee on animal use for research and education at Hallym University (Hallym 2013-2092). A standardized fracture was generated as previously reported by Hiltunen *et al.,* with minor modifications [28, 29]. Mice were anesthetized with intraperitoneal injection of pentobarbital (50 mg/kg) before surgery. A short incision was made on the dorsolateral side of the thigh and was extended over the knee region and a longitudinal incision was made in the patellar tendon. A small 0.5 mm diameter hole was drilled above the tibia tuberosity. Intramedullary fixation was made to stabilize fracture by introducing an Anticorro insect pin into the intramedullary canal of the tibia. The wound was closed by needle, and the same procedure was carried out on the contralateral tibia and on tibiae of all mice used for the experiment. After that, the animal was placed supine under the apparatus fracture apparatus as shown in Figure 1A and the anteromedial diaphysis of the tibia, in the region of the lower midshaft, was exposed to the blunt blade. A weight of 220 g was dropped from a height of 195 mm to produce a closed fracture. Fractures were made in both tibiae of each mouse.

Administration of Kaempferol to Standardized Fracture Model

Twenty male ICR mice were randomly divided into four groups (*n*=5/group) and bilateral fractures were created as described above. The other 5 mice were exposed to a sham surgical procedure without fracture. From the next day after making fractures, Kaempferol (Sigma) in the form of gavage (doses of 0.2, 0.5, 5.0 mg/kg body weight in 20% ethanol) was orally administered every day for 21 days. The vehicle group was given 20% ethanol instead. After 21 days, all mice were sacrificed under anesthesia by cervical dislocation and both tibiae were collected for analysis.

Radiographic Imaging and Histology

Mice tibiae obtained after 21 days were examined
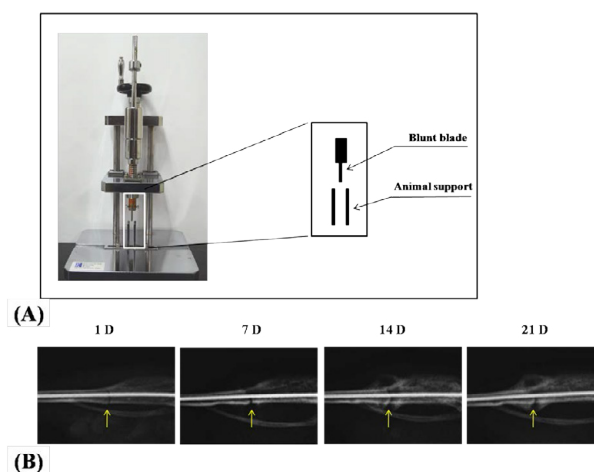
Figure 1. *Establishment of mouse tibia fracture model. Controlled fracture machine used to create a standardized fracture pattern in mouse tibia (A). Radiographical analysis of fracture healing process in mice tibia, which was stabilized by an intramedullary pin (B). Radiographs were taken at 1 day (1D), 7 days (7D), 14 days (14D) and 21 days (21D) after fracture. Arrows indicate fracture sites.*

using X-ray system for radiographic appearance. For microscopic analysis, the tissue at the fracture site was harvested, fixed in 4% paraformaldehyde, decalcified in 20% ethylene-di-amine tetra-acetic acid (EDTA; pH 7.4), and was embedded in paraffin. Sections with 5μm thickness were prepared and stained with hematoxylin and eosin (H&E). All five mice in each group were used for histological analyses.

Measurement of Callus Diameter

Callus dimension was determined at 21 days after euthanization of mice. The fracture site of the tibia was identified by direct dissection and both tibiae were collected. The dimension of the callus was measured in two dimensions; anterior-posterior and lateral-medial using a pocket thickness gage No.7309 (Mitutoyo corp., Japan). The average diameter of the fracture callus was then calculated from these two measurements.

Statistical Analysis

Statistical differences were analyzed by Graphpad Prism 5.0 (San Diego, CA) and evaluated by two-tailed Student's t-test. A p-value less than 0.05 were considered statistically significant.

Results

Radiologic Characterization of Callus Formation During Healing Process

In our study, mice fractures were made transverse or slightly oblique in the lower midshaft of the tibiae by using a standardized fracture generation method and intramedullary fixation was made to facilitate bone healing (materials and methods, and Figure 1A). Radiographic examination revealed good alignment of the fractured tibiae after fixation. Radiographic images taken on day 1, day 7, day 14 and day 21 indicated continuous stages of healing process in mice tibia fractures, in which the external callus formation surrounding the fracture site was noticeably observed. As shown in Figure 1B, a sharp fracture line was seen at day 1, after fracture. At day 7, fracture line became invisible due to early intramembranous ossification. As shown in radiographic images, callus formation was observed at day 14 which became denser at day 21 due to remodeling of cartilaginous callus or endochondral

ossification. However, bone fracture appeared not united even after 21 days (Figure 1B).

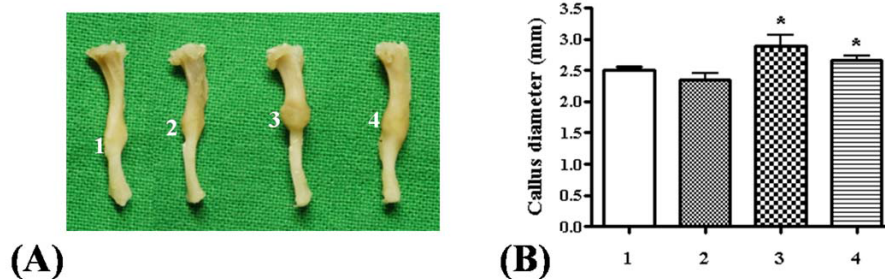### Effect of Kaempferol Treatment on Callus Diameter

Mice were divided into groups as described in materials and methods. At day 21, mice were sacrificed and dissected. The tibiae, which had callus formation, were collected from the hind legs. The representative picture of tibia from each group was taken and an obvious callus formation around the fracture site was detected (Figure 2A). As shown in Figure 2B, significant increase in callus diameter in groups treated with 0.5 and 5 mg/kg of Kaempferol was observed as compared to control group. Specially, the group with 0.5 mg/kg of Kaempferol treatment revealed maximal callus diameter, a significant increment of 15% compared to control group.

### Effect of Kaempferol Treatment on Callus Histology

We next examined the callus histology of tibiae by H&E staining (materials and methods) for endochondral ossification and remodeling phase of bone repair at fracture site. Representative histological sections of the fracture sites on day 21 post-fracture are presented in Figure 3. Histomorphological data displayed that calluses were composed primarily of bone, implicating that most of cartilage matrix was replaced by woven bone after 21 days of fracture induction. It was observed that there is more significant new bone formation in samples from Kaempferol treated groups compared to control group. Dose of 0.5 mg/kg of Kaempferol induced maximal new bone formation (the orange color indicates new bone formation). Histomorphology of callus from 0.5 mg/kg Kaempferol treated group revealed that new bone formation occurs due to enhanced endochondral ossification and initiation of bone remodeling phase with activities of bone cells (nucleus of osteoblasts, osteoclasts and osteocytes as shown by the purple color) (Figure 3B).

### Effect of Kaempferol Treatment on Callus Morphology

Treatment of 0.5 mg/kg of Kaempferol generated maximal callus diameter and largest area of new bone formation. To determine whether an increase in callus diameter and new bone formation are associated with early period of healing process of fracture, the group treated with 0.5 mg/

Figure 2. *Effect of different dose of Kaempferol on callus formation in mice tibia fractures*
(A-B) Representative picture of tibia with external morphology of callus and callus diameter measurement in each group (1: control group; 2: administration of Kaempferol at 0.2 mg/kg; 3: Administration of Kaempferol at 0.5 mg/kg; 4: Administration of Kaempferol at 5 mg/kg) (*p<0.05)
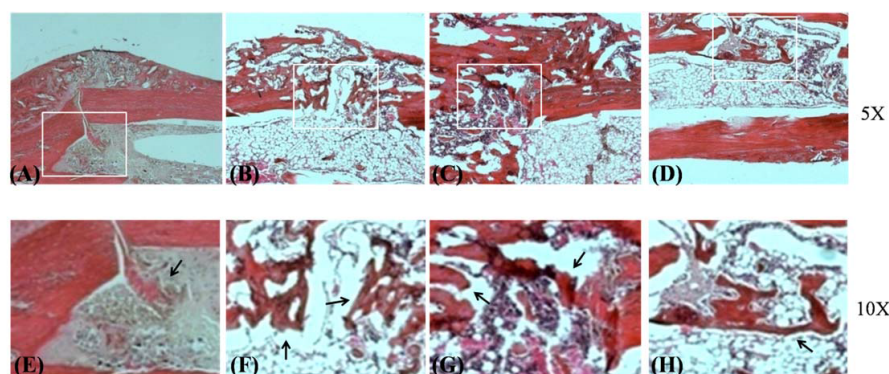
Figure 3. *Comparison of histology of callus in different mice groups by H&E staining*
(A,E) control group; (B,F) administration of Kaempferol at 0.2 mg/kg; (C,G) administration of Kaempferol at 0.5 mg/kg; (D,H)
administration of Kaempferol at 5 mg/kg. Arrows indicate new bone formation at fracture sites.
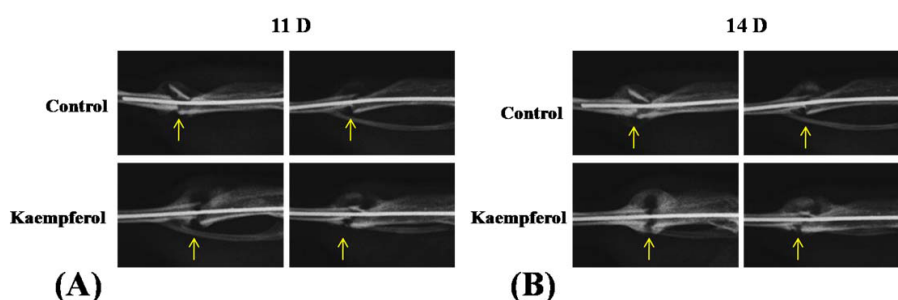
Figure 4. *Comparison of radiography imaging between Kaempferol treated group and control group during fracture healing.*
(A-B) Radiographs were taken at 11th day (11D) and 14th day (14D) post- fracture. Arrows indicate fracture sites

kg of Kaempferol was examined at early time points (11 and 14 days) with respect to control group. Radiographs (Figures 4A and 4B), taken at two time points indicate that calluses from the treated group appeared both larger and denser than calluses from control group. Radiographs of tibiae in control group showed no visible callus formation at 11 days of fracture healing; however in the treated group early callus formation was recorded. After 14 days, calluses from the group with Kaempferol treatment appeared denser and bigger in size than that from control group. Taken together, data indicates that treatment with Kaempferol can induce enhanced early healing of the fractured site compared to control.

Discussion

Bone fracture is a major health issue faced by people of almost all countries. Recovery procedure, known as fracture healing, is a complex process driven by many factors such as cell activities, cytokines, growth factors etc. and is responsible for bone union at fracture site. Occasionally, delayed union or nonunion of bone occurs due to impaired fracture healing. Rates of bone nonunion vary with an average of 5 to 10% in different types of fractures and tibia shaft fracture is a common fracture with high rate of nonunion up to 18.5% [30]. For managing fracture healing, clinically there are two major strategies. First is the operative method which utilizes bone grafts as implants for fixation and augment bone healing. Second strategy is to utilize therapeutic agents to enhance rate and quality of bone healing process. In the aspect of therapeutic drugs, there is no registered agent to deal with fracture healing, till date. Currently, researchers are trying to find potential drug candidates for stimulating bone healing from natural sources due to the safety and economic benefits associated with them. Numerous bioactive compounds have been reported to have positive effects on osteogenesis, osteoblast function and bone health such as phytoestrogen; Genistein, Quercetin, Kaempferol and Naringin [19, 31, 32, 33]. Therefore, taking into consideration of the advantages and positive effect possessed by natural molecules on bone healing, we have tried to observe the effect of natural flavonol, Kaempferol on fracture healing process.

Kaempferol is a promising flavonoid that has been studied for its effect on osteogenesis and bone formation. Kaempferol induces osteogenesis in human primary mesenchymal stem cells as well in rat primary osteoblasts and bone formation in newborn rat calvaria [23, 25, 26]. Moreover, Kaempferol has been shown to stimulate chondrogenic differentiation process and cartilage formation [27]. For the first time, our study demonstrates the positive effect of Kaempferol on fracture healing. The effect of Kaempferol was examined by using a standardized mice fracture model. Kaempferol treatment to mice with fractured tibia showed increased callus size as well as stimulated callus formation during early healing process of fracture. Tibiae collected after 21 days

from two groups either treated with 0.5 mg/kg or 5 mg/kg of Kaempferol had callus diameter significant larger than that from control group. Specifically, group with 0.5 mg/kg of Kaempferol treatment had maximal diameter of callus with an increment of 15% compared with control. The other evidence for enhanced bone healing comes from histomorphological analysis where callus at 21 days post-facture showed an increase in new bone formation in all treated groups compared with control. Group with Kaempferol treatment of 0.5 mg/kg demonstrated maximal bone formation. From accumulating studies including ours, it may be hypothesized that Kaempferol has the ability to induce callus formation and callus remodeling during fracture healing. This effect may at least involve increase in osteogenic activity of mesenchymal stem cells and osteoblasts to form hard callus or may potentiate chondrogenic activity to form soft callus. In other words, recruitment of mesenchymal stem cells and initiation of osteogenic process at fracture site may be stimulated by Kaempferol. Nevertheless, it needs to be verified by more detailed studies elaborating on the signaling mechanism involved for its osteogenic activity. In addition to the effectiveness in promoting bone healing, efficacy and pharmacokinetics of these compounds at clinical level also needs to be elucidated.

Radiographical imaging of calluses from 0.5 mg/kg of Kaempferol treated group appeared both larger and denser than calluses from control group at 11 days and 14 days post-fracture. Callus formation and remodeling of callus are important processes for recovering a fractured site. Our data displayed that Kaempferol at an optimum dose of 0.5 mg/kg affects the callus formation both in term of size as well the initial time needed for healing, and stimulates remodeling of callus by promoting conversion of cartilaginous callus to woven bone. Data obtained here clearly implicates that Kaempferol has a potential to initiate and enhance fracture healing in mice tibia, though, further researches focused on the biomechanical properties of the healed area may be more supportive and require elaborate experimental set up. A view about the microarchitecture and biomechanical properties of fractured site is desirable to predict the bone strength which can help to assess the abilities to perform routine physical activities.

Flavonoids are known to have many good effects on bone health [34, 35]. Recently, a study of Huh *et al*., suggested that formononectin promotes femur fracture healing in rat by increasing angiogenesis and osteogenesis [16]. Similarly, Kaempferol may improve fracture healing by enhancing callus remodeling process for increasing new bone formation. While, phytoestrogens and formononectin have been shown to possess estrogenic effects [36, 37], Kaempferol appears to be more safe as it is reported to possess no estrogenicity at uterine level [17], ruling out any kind of adverse effect due to estrogenic properties.

Taken together, our studies suggest that Kaempferol is a potential therapeutic agent for fracture healing with potential safety and effectiveness associated with it.

Conclusion

Fracture healing process often suffers from nonunion or delayed union due to various clinical abnormalities like osteoporosis. Till date, no effective drugs are available for promoting bone healing in such conditions. Recently a number of phytoestrogens have been shown to promote osteogenesis and bone formation *in vitro* and *in vivo*. In here, we have assessed ability of a flavanol, Kaempferol in potentiating the bone healing process in tibia shaft fracture model of mice. Results obtained demonstrate that Kaempferol enhanced the callus formation and diameter at fracture sites in mice. Moreover, histo-morphological analysis demonstrated recruitment of cells favoring bone formation at fracture site after the treatment of Kaempferol. A dose of 0.5 mg/kg was found to be most effective compared to control. Future studies focused on elucidating the mechanism of action as well as the biomechanical properties of the healed fracture site by Kaempferol can provide more supportive information about the potential of Kaempferol for fracture healing.

Acknowledgements

Conflict of Interest

There is no conflict of interest.

References

1. Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: The cellular picture. Semin Cell Dev Biol 2008; 19: 459-466.

2. Stuermer EK, Sehmisch S, Rack T, Wenda E, Seidlova-Wuttke D. Estrogen and raloxifene improve metaphyseal fracture healing in the early phase of osteoporosis. A new fracture-healing model at the tibia in rat. Langenbecks Arch Surg 2010; 395: 163-172.

3. Lyritis G, Boscainos PJ. Calcitonin effects on cartilage and fracture healing. J Musculoskelet Neuronal Interact 2001; 2: 137-142.

4. Kyllonen L, D'Este M, Alini M, Eglin D. Local drug delivery for enhancing fracture healing in osteoporotic bone. Acta Biomater 2015; 11: 412-434.

5. Kakar S, Einhorn TA, Vora S, Miara LJ, Hon G. Enhanced chondrogenesis and Wnt signaling in PTH-treated fractures. J Bone Miner Res 2007; 22: 1903-1912.

6. Simpson AH, Mills L, Noble B. The role of growth factors and related agents in accelerating fracture healing. J Bone Joint Surg Br 2006; 88: 701-705.

7. Moroni A, Faldini C, Hoang-Kim A, Pegreffi F, Giannini S. Alendronate improves screw fixation in osteoporotic bone. J Bone Joint Surg Am 2007; 89: 96-101.

8. Gerstenfeld LC, Sacks DJ, Pelis M, Mason ZD, Graves DT. Comparison of effects of the bisphosphonate alendronate versus the RANKL inhibitor denosumab on murine fracture healing. J Bone Miner Res 2009; 24: 196-208.

9. Glass GE, Jain A. Cochrane corner: bone morphogenetic protein (BMP) for fracture healing in adults. J Hand Surg Eur Vol 2013; 38: 447-449.

10. Bigham-Sadegh A, Oryan A. Basic concepts regarding fracture healing and the current options and future directions in managing bone fractures. Int Wound J 2015; 12: 238-247.

11. Brandi ML. Drugs for bone healing. Expert Opin Investig Drugs 2012; 21: 1169-1176.

12. Hsueh TP, Chiu HE. Traditional Chinese medicine speeds-up humerus fracture healing: two case reports. Complement Ther Med 2012; 20: 431-433.

13. Mohammad S, Pal US, Pradhan R, Singh N. Herbal remedies for mandibular fracture healing. Natl J Maxillofac Surg 2014; 5: 35-38.

14. Chow SP, Yeung HW, Law LK, Chan TM, Lau C. The effect of Davallina Orientalis on bone healing--a preliminary report. Am J Chin Med 1982; 10: 101-106.

15. Ozturk A, Ilman AA, Saglam H, Yalcinkaya U, Aykut S. The effects of phytoestrogens on fracture healing: experimental research in New Zealand white rabbits. Ulus Travma Acil Cerrahi Derg 2008; 14: 21-27.

16. Huh JE, Kwon NH, Baek YH, Lee JD, Choi DY. Formononetin promotes early fracture healing through stimulating angiogenesis by up-regulating VEGFR-2/Flk-1 in a rat fracture model. Int Immunopharmacol 2009; 9: 1357-1365.

17. Trivedi R, Kumar S, Kumar A, Siddiqui JA, Swarnkar G. Kaempferol has osteogenic effect in ovariectomized adult Sprague-Dawley rats. Mol Cell Endocrinol 2008; 289: 85-93.

18. Miyake M, Arai N, Ushio S, Iwaki K, Ikeda M. Promoting effect of Kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1. Biosci Biotechnol Biochem 2003; 67: 1199-1205.

19. Prouillet C, Maziere JC, Maziere C, Wattel A, Brazier M. Stimulatory effect of naturally occurring flavonols quercetin and Kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. Biochem Pharmacol 2004; 67: 1307-1313.

20. Kumar A, Gupta GK, Khedgikar V, Gautam J, Kushwaha P. In vivo efficacy studies of layer-by-layer nano-matrix bearing Kaempferol for the conditions of osteoporosis: a study in ovariectomized rat model. Eur J Pharm Biopharm 2012; 82: 508-517.

21. Lee WS, Lee EG, Sung MS, Yoo WH. Kaempferol inhibits IL-1beta-stimulated, RANKL-mediated osteoclastogenesis via downregulation of MAPKs, c-Fos, and NFATc1. Inflammation 2014; 37: 1221-1230.

22. Wattel A, Kamel S, Mentaverri R, Lorget F, Prouillet C. Potent inhibitory effect of naturally occurring flavonoids quercetin and Kaempferol on in vitro osteoclastic bone resorption. Biochem Pharmacol 2003; 65: 35-42.

23. Yang L, Takai H, Utsunomiya T, Li X, Li Z. Kaempferol stimulates bone sialoprotein gene transcription and new bone formation. J Cell Biochem 2010; 110: 1342-1355.

24. Suh KS, Choi EM, Kwon M, Chon S, Oh S. Kaempferol attenuates 2-deoxy-d-ribose-induced oxidative cell damage in MC3T3-E1 osteoblastic cells. Biol Pharm Bull 2009; 32: 746-749.

25. Guo AJ, Choi RC, Zheng KY, Chen VP, Dong TT. Kaempferol as a flavonoid induces osteoblastic differentiation via estrogen receptor signaling. Chin Med 2012; 7: 10.

26. Byun MR, Jeong H, Bae SJ, Kim AR, Hwang ES. TAZ is required for the osteogenic and anti-adipogenic activities of Kaempferol. Bone 2012; 50: 364-372.

27. Nepal M, Li L, Cho HK, Park JK, Soh Y. Kaempferol induces chondrogenesis in ATDC5 cells through activation of ERK/BMP-2 signaling pathway. Food Chem Toxicol 2013; 62: 238-245.

28. Hiltunen A, Vuorio E, Aro HT. A standardized experimental fracture in the mouse tibia. J Orthop Res 1993; 11: 305-312.

29. Carmouche JJ, Puzas JE, Zhang X, Tiyapatanaputi P, Cory-Slechta DA, Gelein R, Zuscik M, Rosier RN, Boyce BF, O'Keefe RJ. Lead exposure inhibits fracture healing and is associated with increased chondrogenesis, delay in cartilage mineralization, and a decrease in osteoprogenitor frequency. Environ Health Perspect 2005; 113: 749-755.

30. Fong K, Truong V, Foote CJ, Petrisor B, Williams D. Predictors of nonunion and reoperation in patients with fractures of the tibia: an observational study. BMC Musculoskelet Disord 2013; 14: 103.

31. Trivedi R, Kumar A, Gupta V, Kumar S, Nagar GK. Effects of Egb 761 on bone mineral density, bone microstructure, and osteoblast function: Possible roles of quercetin and Kaempferol. Mol Cell Endocrinol 2009; 302: 86-91.

32. Heim M, Frank O, Kampmann G, Sochocky N, Pennimpede T. The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells. Endocrinology 2004; 145: 848-859.

33. Wong RW, Rabie AB. Effect of naringin on bone cells. J Orthop Res 2006; 24: 2045-2050.

34. Taku K, Melby MK, Takebayashi J, Mizuno S, Ishimi Y. Effect of soy isoflavone extract supplements on bone mineral density in menopausal women: meta-analysis of randomized controlled trials. Asia Pac J Clin Nutr 2010; 19: 33-42.

35. Welch AA, Hardcastle AC. The effects of flavonoids on bone. Curr Osteoporos Rep 2014; 12: 205-210.

36. Santell RC, Chang YC, Nair MG, Helferich WG. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. J Nutr 1997; 127: 263-269.

37. Mu H, Bai YH, Wang ST, Zhu ZM, Zhang YW. Research on antioxidant effects and estrogenic effect of formononetin from Trifolium pratense (red clover). Phytomedicine 2009; 16: 314-319.

Correspondence to:

Ju-Suk Nam, PhD
Institute for Skeletal Aging & Orthopedic Surgery
Hallym University Hospital-College of Medicine
Chuncheonsi
Gangwon-do 24252
Republic of Korea

Sang-Soo Lee MD, PhD
Institute for Skeletal Aging & Orthopedic Surgery
Hallym University Hospital-College of Medicine
Chuncheon-si
Gangwon-do 24252
Republic of Korea