Enhanced healing process of fracture by treatment of Kaempferol.

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Abstract

Recently, plant-derived phytoestrogens have been shown to possess promising results for bone preservation and bone augmentation. Kaempferol, one of flavans, 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 tibias 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.

Keywords: Kaempferol, Phytoestrogen, Flavonoids, Fracture healing, Mouse tibia fracture model.

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].
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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 has been reported to promote osteogenic differentiation in murine osteoblasts [25], murine mesenchymal cell line and human primary mesenchymal stem cells [26]. 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 osteoelastic 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 Anticorros 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...
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/kg 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/kg Kaempferol generated maximal callus diameter and largest area of new bone formation.

![Figure 2. Effect of different dose of Kaempferol on callus formation in mice tibia fractures](image)
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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-facture. 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 formononetin 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 formononetin 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.

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**Conflict of Interest**

There is no conflict of interest.

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