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# Effect of naringin collagen graft on bone formation

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**Abstract**

Naringin is a flavonoid available commonly in citrus fruits and is also a HMG-CoA reductase inhibitor. Our laboratory compared the amount of new bone produced by naringin in collagen matrix to that produced by bone grafts and collagen matrix. Twenty bone defects, 5 mm × 10 mm were created in the parietal bone of 14 New Zealand White rabbits. In the experimental group, 5 defects were grafted with naringin solution mixed with collagen matrix, 5 defects were grafted with autogenous endochondral bone. In the control groups, 5 defects were grafted with collagen matrix alone (active control) and 5 were left empty (passive control). Animals were killed on day 14 and the defects were dissected and prepared for histological assessment. Serial sections were cut across each defect. Quantitative analysis of new bone formation was made on 150 sections (50 sections for each group) using image analysis. A total of 284% and 490% more new bone was present in defects grafted with naringin in collagen matrix than those grafted with bone and collagen, respectively. No bone was formed in the passive control group. In conclusion, naringin in collagen matrix have the effect of increasing new bone formation locally and can be used as a bone graft material.


*Keywords:* Bone repair; Bone graft; Collagen matrix; BMP; Naringin

## 1. Introduction

To develop materials to replace lost bone, osteogenic matrices such as demineralized bone matrix (DBM) have been extensively studied [1–4] since they were reported by Urist [5]. Our laboratory has shown that DBM derived from intramembranous bone has extremely good osteogenic properties and greatly improved the integration of autogenous bone grafts in rabbit skulls [6]. However, the risk of transmission of infection and immunological reaction prompted scientists to continue to search for new materials that increase bone formation, especially from using plant or synthetic materials [7].

Bone morphogenetic proteins (BMPs), the active components of DBM, are important regulators in osteogenic differentiation during fracture repair [8–10]. Wang et al. [11] showed that BMP-2 caused commitment and differentiation of multipotential stem cell line into osteoblastlike cells. To discover small molecules that induce BMP-2, Mundy et al. [12] examined more than 30 000 compounds from a collection of natural products and tested the effects of compounds on BMP-2 gene expression. They identified that statin, a common cholesterol-lowering drug that inhibits hepatic hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway, as the only natural product in that collection that specifically increased expression of the BMP-2 gene. Sugiyama et al. [13], using real time polymerized chain reaction analysis and alkaline phosphatase assay revealed that the statin compactin induced an increase in the expression of BMP-2 mRNA and protein. Like compactin, simvastatin also activated the BMP-2 promoter, whereas pravastatin did not [13]. Oophorectomized rats given statins in oral dosages, comparable with those used in humans (that is, 1–10 mg/kg per day of simvastatin), had a 40–90% increase in the trabecular bone volume of the femur and lumber vertebrae within 35 days, relative to rats given placebo [14]. Skoglund et al. [15] showed that high-dose systemic treatment with simvastatin improved fracture healing in a mice femur fracture model. To examine the ability of statin to be used clinically for the

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repair of bone defects, our laboratory have examined its osteogenicity in the presence of a carrier in an animal study [16]. Our laboratory showed that 308% more new bone was formed in defects grafted with statin mixed with a collagen matrix carrier than those grafted with the collagen matrix carrier alone in 14 days. Unfortunately, statin also has some side effects such as digestive disturbances, weakness, headache and possibility of serious muscle problems [17], making the search for an ideal bone inductive agent still a necessity.

Further studies showed that the statin-mediated activation of BMP-2 promoter was completely inhibited by the downstream metabolite of HMG-CoA reductase, mevalonate, indicating that the activation was a result of the inhibition of that enzyme [13]. Therefore, it is possible that any drug that inhibits the HMG-CoA reductase may have the similar effect of statin in the activation of the BMP-2 promotor.

Naringin is a polymethoxylated flavonoid commonly found in citrus fruits. Research on it has been focused on its antioxidant and anticholesterol effect. It was also shown to have a HMG-CoA reductase inhibiting effect [18]. Therefore, it is possible that naringin may also activate the BMP-2 promotor and increase the bone formation. The significance is, naringin is present in large amount in common edible fruits like grapefruit. Therefore, if naringin can be shown to increase bone in an animal model of bone defect healing, it may be the long-sought-after safe agent for bone induction and bone defect repair. In addition, it also increased our understanding in the relationship between the mevalonate pathway and the bone forming pathway.

In the present study our laboratory decided to examine its bone forming ability in vivo for the repair of bone defects by using a carrier to enable its use in the clinical setting. To achieve this, our laboratory measured the amount of new bone produced by naringin with collagen matrix carrier grafted into bony defects and compared with that of autogenous endochondral bone graft alone and that of collagen matrix alone.

## 2. Materials and methods

### 2.1. Experimental and control groups

Twenty $10 \times 5$ mm full-thickness bone defects were created in the parietal bones of 14 New Zealand White rabbits from an inbred colony. The rabbits were 5 months old (adult stage) and weighed 3.5–4.0 kg. The handling of the animals and the experimental protocol were approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. In the experimental groups, 5 defects were grafted with naringin solution mixed with collagen matrix (naringin group) and 5 defects were grafted with autogenous endochondral bone (bone graft group). In the control groups, 5 defects were grafted with collagen matrix alone (positive control) and 5 were left empty (negative control). The sample size was based on previous research using this model. Our laboratory use a small sample size because there was a large difference in bone formation between different groups so that statistical significant difference can be detected with minimal number of animals, also from the animal ethic committee that the smallest sample size that allows a significant difference to be detected should be used.

For the naringin group and the control groups, two defects were created on the parietal bone of each rabbit. In each group six defects were created and surgery performed but after sacrifice only five was randomly drawn and prepared for analyses. For the bone graft group, one defect was created on the left parietal bone because one defect was created on the right diaphyseal tibial shaft during harvesting of the bone graft, the rationale was to ensure the surgical insult (two bone defects in each rabbit) was similar for different groups.

### 2.2. Surgical procedures

The details of the operation and the postoperative care of the animals were previously described [19]. In short, the animals were premedicated 1 h before surgery with oxytetracycline hydrochloride (200 mg/ml, 30 mg/kg body weight, Tetroxyla, Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3 ml/kg body weight, Hypnorm, Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5 mg/ml, 1 mg/kg body weight, Valium 10, Roche). In order to maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1 ml/kg) were given at 30-min intervals during the operation.

The surgical procedure consisted of the creation of one or two $10 \times 5$ mm full-thickness (approximately 2 mm) cranial defects, devoid of periosteum, using templates, in the parietal bones (Fig. 1). The defects were produced using round stainless steel bars (1 mm) in diameter) on a low speed dental drill. Outlines of the defects were made initially by making holes of full thickness the parietal bone using a stainless steel wire template bent to the required size of the defect. The holes were joined to complete the process. During the cutting of bone, copious amount of sterile saline was used for irrigation and to minimize thermal damage to the tissues. Depending on which groups in which the rabbit belonged, the defects were grafted with different materials. In the experimental group, the defects were filled with naringin in collagen matrix which consisted of 0.2 ml naringin solution (MERCK & CO, Inc., NJ, USA, dissolved in

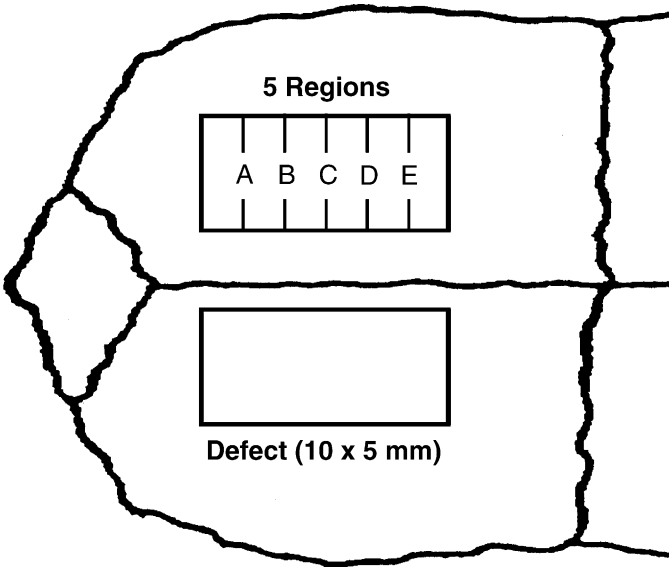


Fig. 1. Diagram of the dorsal view of the skull of a rabbit, with the anterior region orientated to the right, showing the sites of two surgically created bone defects on the parietal bones and five regions within the defect from which sections were taken for quantitative analyses. From more than 10 sections cut in each region, 2 sections were selected randomly and measured for area new bone formed, giving a total of 10 sections from each defect. Therefore, the amount of new bone formation was assessed throughout the whole defect.