Effect of genistin on bone formation

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1. ABSTRACT

The amount of new bone produced by grafting genistin in collagen matrix was compared to the bone produced by collagen matrix alone. A total of 18 bone defects (5 mm by 10 mm) were created in the parietal bone of 9 New Zealand White rabbits. Six defects were grafted with genistin mixed with collagen matrix, while 6 were grafted with collagen matrix alone (positive control) and 6 were left empty (negative control). Animals were killed on day 14 and defects were prepared histologically. Quantitative analysis of new bone formation and bone cells was carried out on 100 sections (50 sections for each group) using image analysis. A total of 520% more new bone was present in defects grafted with genistin in collagen matrix than in defects grafted with collagen matrix alone ($p<0.0001$), while no bone was formed in the negative control group. The amount of bone forming osteoblasts was also significantly greater in the genistin group than the positive control group. Therefore, genistin in collagen matrix can increase new bone formation locally *in vivo*.

2. INTRODUCTION

Bone induction is needed for treatment of bone trauma and for filling osseous defects, as part of some surgical procedures. Autogenous cancellous bone grafting has become the gold standard for replacing bone (1), but its major limitations are inadequate performance and surgical morbidity such as donor site pain, paresthesia and infection. The morbidity rate can be as high as 8-10% (2). Moreover, graft resorption poses a severe problem; in an experimental study, endochondral bone grafts showed a volume loss of 65% (3). Although allografts are an alternative to autogenous grafts, they seem to be biologically inferior and are associated with infection and inflammation (4).

The search for chemicals and materials that can increase bone formation would be useful in improving the management of osteoporosis and promoting bone healing after trauma or surgery. The sex hormone estrogen is a possible candidate. Reduced levels of serum estrogen after

menopause were associated with bone loss and osteoporosis, and estrogen replacement therapy has been used successfully to prevent bone loss (5). Furthermore, *in vitro* studies revealed that estrogen enhanced osteoblast differentiation and bone formation (6), and inhibited osteoclast development (7).

Phytoestrogens, plant-derived non-steroidal compounds, are also possible candidates. They bind to estrogen receptors (ERs) and have estrogen-like activity (8). They have attracted much attention due to their potential role in preventing and treating cardiovascular diseases, osteoporosis, diabetes, obesity, menopausal symptoms, renal diseases and various cancers (9,10). Phytoestrogens are divided into three classes: isoflavones, coumestans and lignans. Some flavonoids, such as flavonols, are also known as isoflavone phytoestrogens (10), and their interactions with both ERa and ERb subtypes of ER are well documented (11). Because osteoblasts express both ERa and ERb receptors (12), flavonols and isoflavones may be able to modify osteoblast activity and hence bone formation.

Genistin is a flavonoid belonging to the isoflavone class of phytoestrogens. Genistin and its aglycone genistein are found mainly in legumes such as soybeans, and most soy foods contain about 1 to 2 mg of genistin per gram of protein (13). Findings from previous studies suggest that genistin and genistein have estrogen-like effects. In Asia, populations that eat soy foods regularly and whose daily diet contains 20 to 80 mg of genistin have lower rates of breast and prostate cancer than groups in the West, which have less genistein in their diets (13). In addition, animal studies and double-blind, placebo-controlled human trials showed that genistein could help restore bone protection (14-17). Interestingly, unlike estrogen, which primarily helps prevent the destruction of bone, evidence indicate that genistein may also assist in creating new bone (14,16). Because genistin is present in large amounts of daily diets in Asia, if it increases bone formation in animal model of bone defect healing, then it may represent an attractive, safe, accessible and effective agent for bone induction and bone defect repair. Although there were studies on the systemic effect of genistin on bone, there were no studies on the local effect of genistin on bone. If genistin stimulates bone formation locally, it will be useful in a number of clinical aspects like production of bone graft materials, promotion of bone defect and fracture healing and application in bone regeneration, especially in cases of compromised host response like after irradiation. It also opens a new research area on bone tissue engineering using phytoestrogens.

In the present study, we therefore examined the local bone-forming ability of genistin during repair of bone defects *in vivo*. We measured the amount of new bone produced by genistin when grafted with collagen matrix carrier into defected bone in rabbits, and compared the amount of new bone with that produced after grafting of collagen carrier alone.

3. MATERIALS AND METHODS

3.1. Animals

The protocol of Wong and Rabie (18) was followed. Two 10- by 5-mm full-thickness (approximately 2 mm) bone defects were created in the parietal bone of each of 9 New Zealand White rabbits from an inbred colony. The rabbits were 5 months old (adult stage) and weighed 3.5 to 4.0 kg. The handling of the animals and the experimental protocol were approved by the local ethical Committee of The University of Hong Kong. In the experimental group, 6 defects were grafted with genistin solution mixed with collagen matrix as a carrier. In the control groups, 6 defects were grafted with collagen matrix alone (positive control) and 6 were left empty (negative control). The sample sizes were selected as previously reported (18). At the end of the grafting period, 5 defects per group were randomly selected and prepared for analyses.

3.2. Surgical procedure

The animals were premedicated 1 hour prior to surgery with oxytetracycline hydrochloride (200 mg/mL, 30 mg/kg body weight of Tetroxyla; Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3 mL/kg body weight of Hypnorm; Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5 mg/mL, 1 mg/kg body weight of Valium 10; Roche, Basal, Switzerland). To maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1 mL/kg) were given at 30-minute intervals during the operation. The surgical procedure consisted of the creation of a total of eighteen 10- by 5-mm full-thickness cranial defects devoid of periosteum in the parietal bone of 9 rabbits (Figure 1). The defects were produced by making holes around the edge of a stainless-steel wire template with a round stainless-steel bur (1 mm in diameter) attached to a low-speed dental drill. The holes were then gradually joined up. During the cutting of the bone, copious amounts of sterile saline were used for irrigation and to minimize thermal damage to the tissues.

Based on results from a pilot study, defects in the experimental group were filled with 0.2 mL solution of genistin (Sigma-Aldrich, MO, USA) that had been dissolved in water to a concentration of 100 mg/mL and mixed with 0.02 g of purified absorbable fibrillar collagen (Collagen Matrix Inc, NJ, USA). The grafts were prepared 15 minutes prior grafting. Positive controls were grafted with 0.02 g of collagen matrix mixed with 0.2 mL water, whereas negative controls were left empty.

3.3. Postoperative care

All wounds were closed with interrupted 3/0 black silk sutures. No attempt was made to approximate the periosteum to prevent the barrier effect. Postoperatively, the rabbits were given oxytetracycline hydrochloride daily for 10 days and buprenorphine hydrochloride for 2 weeks. Two weeks after surgery, the animals were killed using sodium pentobarbitone and bone defects as wells as surrounding tissue were immediately collected for histological analysis
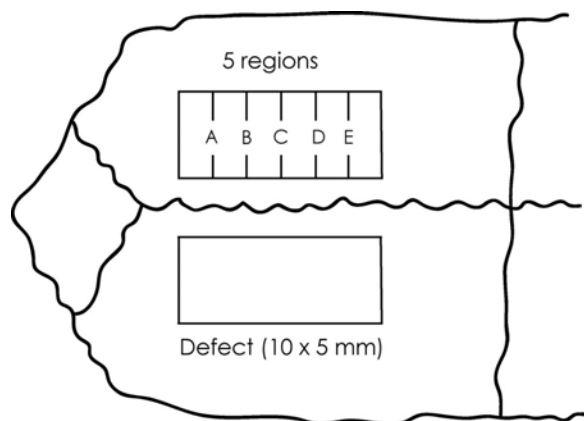
Figure 1. Diagram of dorsal view of rabbit skull, showing the sites of two surgically created bone defects.
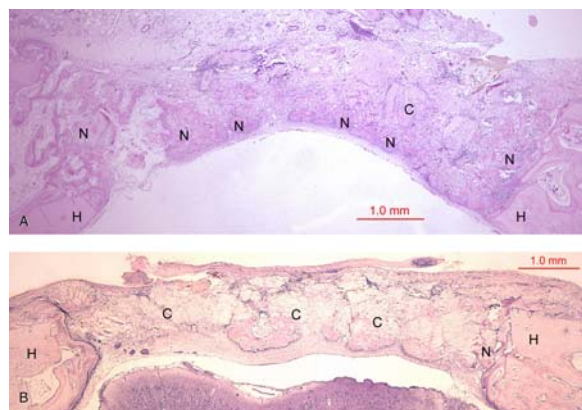

Figure 2. A. Photomicrograph showing healing of a bony defect grafted with genistin in collagen matrix on day 14. New bone (N) can be seen spanning the defect. H = host bone. Some collagen matrix (C) remained at the centre of the bony defect. (Periodic acid-Schiff stain; original magnification, x40.) B. Photomicrograph of defect grafted with collagen matrix (positive control) on day 14. No bone could be seen across the defect except for a little new bone (N) near the ends of the host bone (H). Collagen matrix (C) remained across the bone defect. (Periodic acid-Schiff stain; original magnification, x40.)

3.4. Histological preparation and analysis

Tissues were fixed in 10% neutral buffered formal saline solution, demineralized with K's Decal Fluid (sodium formate/formic acid) and double-embedded in celloidin-paraffin wax. Each tissue sample containing the whole defect was embedded intact, and serial sections of 5-μm thickness containing the defect were cut perpendicular to the long axis and subjected to quantitative analysis.

Defects were divided into 5 regions spaced at 1500 μm apart (Figure 1) and 2 serial sections were selected randomly for each region, giving a total of 10 representative samples along the length of each defect. Five defects in each group were tested, and a total of 50 sections per group were analyzed. The total amount of new bone formed within the surgically created defects was measured

as previously described by Wong and Rabie (18,19). Briefly, samples were stained with periodic acid-Schiff stain, and images from transmitted-light microscopy (Leica, Solms, Germany) were captured and digitized by a video camera (Single CCD Color Camera Tk-C1380E; JVC, Tokyo, Japan). One observer, analyzed coded samples, and outlined the periphery of the newly formed bone at the graft-host interface on the computer image, aided by the clear difference in staining and morphology between newly formed bone and mature bone (new bone was denoted "N"; Figures 2 and 3). The computer image analysis system and software (Leica Qwin Image Processing and Analysis Software v2.3; Leica Microsystems Imaging Solutions Ltd, Solms, Germany) were then used to calculate the total area of the outlined new bone. As a reliability test, 10 randomly selected histological sections were digitized on two separate occasions at least three months apart by the same observer and also by an independent observer. The number of bone forming osteoblast and bone resorbing osteoclast, on newly formed bone between defects grafted with genistin and collagen matrix and that of defects grafted with collagen matrix only, were also counted on all the slides that had the amount of new bone measured.

3.5. Statistical analyses

Data were statistically analyzed using software Graphpad Instat v2.04a; 2000 (San Diego, USA). One-way analysis of variance (ANOVA) was used to compare sections from the five regions in each defect. The arithmetic mean amount of new bone, standard deviation (SD) and 95% confidence intervals were calculated for each group. The means of the genistin group and positive control group were compared by Welch's unpaired t-test, which does not assume equal variances, with $p<0.05$ chosen as the critical level of statistical significance.

The size of the method error in digitizing the areas of new bone was 0.014 mm^2, as calculated by the formula where method error equals to the square root of (the sum of the squared differences between the two sets of two mean values over the double of the number of double measurements). Two-tailed paired t-tests were also performed to compare intraobserver and interobserver registrations. The p value comparing intra-observer registrations was 0.5652, and the p value to compare inter-observer registrations was 0.5911; both were considered not significant.

4. RESULTS

4.1. Clinical and physical examinations

All animals remained in excellent health throughout the course of the experiment and recovered rapidly from surgery. with no evidence of adverse effects or infection in any of the animals.

4.2. Histological findings

In the group of bone defects grafted with genistin and collagen matrix, integration of the genistin and collagen with the recipient bed was characterized by the presence of new bone. New bone formed at the host bone-

Table 1. Comparison of amounts of new bone (mm^2) in five defects grafted with genistin in collagen matrix

Region	Genistin Defect 1	Genistin Defect 2	Genistin Defect 3	Genistin Defect 4	Genistin Defect 5
A	1.82	1.25	2.22	2.47	4.62
	2.24	1.56	2.59	2.65	4.80
B	1.92	1.32	2.24	2.95	3.66
	2.00	1.27	2.34	2.73	2.80
C	2.49	1.64	2.17	1.96	3.92
	2.35	2.22	2.25	1.92	4.20
D	3.13	2.12	1.59	1.94	4.01
	3.24	2.07	2.08	2.05	3.90
E	2.75	1.91	1.66	2.84	3.83
	2.71	1.79	1.67	2.87	4.04
Mean	2.465	1.715	2.081	2.438	3.978
SD	0.4919	0.3634	0.3324	0.4265	0.5439
df	4,5	4,5	4,5	4,5	4,5
F	23.945	5.326	5.068	41.647	6.091
P	0.0019	0.0477	0.0523	0.0005	0.0368

Mean and standard deviation (SD) of 10 sections of each defect, and results of ANOVA (degrees of freedom [df], F, P) comparing 5 regions within each defect

Table 2. Comparison of amounts of new bone (mm^2) in five defects grafted with collagen matrix (positive control)

Region	Control Defect 1	Control Defect 2	Control Defect 3	Control Defect 4	Control Defect 5
A	0.68	0.64	0.18	0.12	0.41
	0.74	0.82	0.18	0.11	0.42
B	0.57	0.68	0.15	0.31	0.29
	0.51	0.71	0.16	0.38	0.34
C	0.70	0.85	0.35	0.23	0.31
	0.70	0.96	0.32	0.18	0.35
D	0.58	0.86	0.20	0.22	0.07
	0.74	0.95	0.21	0.24	0.08
E	0.60	0.51	0.06	0.15	0.11
	0.80	0.62	0.06	0.13	0.09
Mean	0.662	0.76	0.187	0.145	0.247
SD	0.09223	0.1503	0.09393	0.07412	0.1431
df	4,5	4,5	4,5	4,5	4,5
F	1.379	6.494	179.23	34.071	96.734
P	0.3603	0.0324	<0.0001	0.0008	<0.0001

Mean and standard deviation (SD) of 10 sections of each defect, and results of ANOVA (degrees of freedom [df], F, P) comparing 5 regions within each defect

graft interface and tended to grow across the defect (Figure 2A); no cartilage was found. At higher magnification (Figure 3), new bone could be seen spanning from host bone across the defect, and growing towards and amalgamating with the collagen matrix. Bone cells like osteoblasts and osteocytes were present, confirming that the collagen had not just calcified and new bone had formed, capillaries were also present.

In the positive control group, little new bone formed at the host bone-graft interface, and some collagen fibers were present at the center of the defects (Figure 2B). In the negative control group, the defect had healed and fibrous tissue bridged the defect, but very little new bone had formed at the ends of the host bone, so no quantitative analysis was performed.

4.3. Quantitative analysis

A total of 50 sections each in the experimental group and the positive control group were digitized and analyzed. The amount of newly formed bone was significantly greater in the defects grafted with genistin in collagen matrix than in those grafted with collagen matrix only (Tables 1 and 2; Figure 3). In the experimental group, the mean area of newly formed bone per defect was 2.54 mm^2 (SD, 0.8854 mm^2). In the positive control group, the mean area was 0.41 mm^2 (SD, of 0.2728 mm^2). These two

values were statistically significant ($p<0.0001$). The mean number of bone forming osteoblast on newly formed bone between defects grafted with genistin solution and collagen matrix, and that of defects grafted with collagen matrix only were 760 and 172, respectively (Table 3). The mean number of bone resorbing osteoclast on newly formed bone between defects grafted with genistin solution and collagen matrix, and that of defects grafted with collagen matrix only were 6 and 14, respectively. Because of the small number, statistical testing was not performed.

5. DISCUSSION

In vivo supplementation of genistin in collagen matrix significantly increased new bone formation locally when grafted into skull defects, producing 520% more new bone than the absorbable collagen sponge alone (Figure 4). In addition, the new bone, not only formed near the margin of the defect but also at the center, which is a rare scenario with other osteogenic agents. A little bone formation was observed in the defects in the control groups. The defects were mostly repaired with fibrous tissue within a short experimental period (two weeks) of time as depicted in Figure 2B. Ultimately these defects would be repaired with the bone but it would take few months (Figure 5). Our findings show that genistin is osteogenic when used with collagen matrix and are consistent with those of *in vitro*

Table 3. Comparison of mean (SD) number of osteoblast on newly formed bone between defects grafted with genistin solution and collagen matrix and that of defects grafted with collagen matrix only

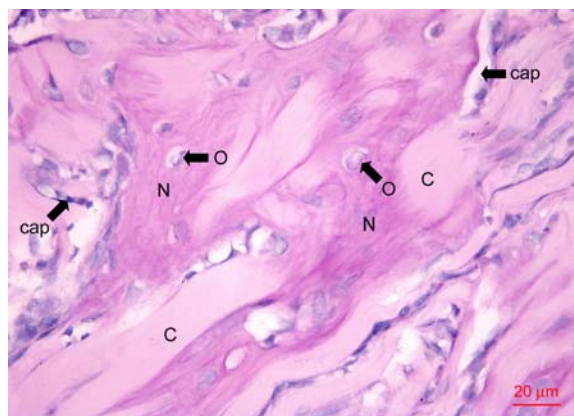| Group		P value
Genistin	760 (256)	< 0.0001
Control	172 (89)	

Figure 3. High-power photomicrograph showing the formation of new bone in a bony defect grafted with genistin in collagen matrix. New bone (N) and osteocytes (O) could be seen growing around the collagen matrix (C). Capillaries (cap) were also present. No cartilage was found. (Periodic acid-Schiff stain; original magnification, x200.)
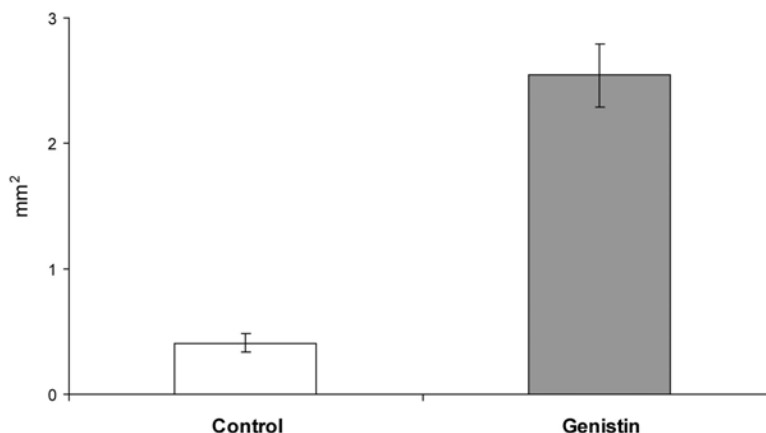

Figure 4. Comparison of mean areas of newly formed bone between (A) defects grafted with collagen matrix (0.41 mm^2, SD =0.2728 mm^2) and (B) defects grafted with genistin in collagen matrix (2.54 mm^2, SD=0.8854 mm^2). Welch's unpaired t test, (p<0.0001).

studies demonstrating that genistin is one of the most potent osteogenic tested chemicals so far (18,20-23).

The rabbit model used in this study was relevant because non-grafted control bone defects did not heal within two weeks period after their creation, whereas two weeks was sufficient to show healing in the experimental graft group. Furthermore, grafting with collagen matrix alone achieved minimal bone healing across the defect in the positive control group (Figure 2B). The grafting protocol itself had minimal morbidity, as all the rabbits were in good health and condition after the surgery. The 2-week study period to examine bone formation during the early healing of the bone defect was chosen as previously reported (19). It was also the time span chosen for other

studies on bone formation using the same animal model (21,22,25-28), thereby allowing comparisons to be made.

The ANOVA results of the different regions within each defect illustrated the necessity to analyze multiple regions within each defect (Tables 1 and 2). As indicated by the p values, the amount of new bone formed between the various regions within each defect was significantly different in most of the defects. Variability arose because the two sections in each region to be measured were randomly selected among the serial sections. Despite the variations in the amount of bone formed within different regions of different defects, the overall amount of bone formation in the genistin group was greater than that in the control groups.

Table 4. Comparison of mean (SD) number of osteoclast on newly formed bone between defects grafted with genistin solution and collagen matrix and that of defects grafted with collagen matrix only

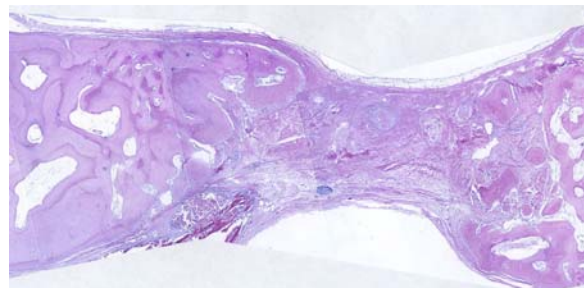| Group	
Genistin	6 (1)
Control	14 (5)

Figure 5. Photomicrograph of defect grafted with collagen matrix (positive control) after 3 months. Note that bone healing occurred and the new bone tended to grow halfway across the defect. (Periodic acid-Schiff stain; original magnification, x40.)

The mean value of bone forming osteoblasts, which is directly related to the amount of bone formation on newly formed bone between defects grafted with genistin and collagen matrix, was significantly greater than that of defects grafted with collagen matrix only (Table 4), indicating that genistin increased bone formation through an increase in osteoblast formation. The mean number of osteoclast, which is directly related to the amount of bone resorption, on newly formed bone of both the defects grafted with genistin and collagen matrix and that of defects grafted with collagen matrix only were small; this was expected at two weeks period of bone defects creation, where active bone healing was taking place. Because of the small number, comparison between groups may not be meaningful.

Collagen matrix (purified absorbable fibrillar collagen) was used in this study because it had been used successfully as a carrier for growth factors such as BMP-2 to induce bone formation in animals and in humans (24). It is derived from bovine tendon in the fibrillar form, and the manufacturer (Collagen Matrix Inc, NJ, USA) suggested its usefulness for the delivery of cells and growth factors as well as for gene therapy. It has also been successfully used with rhBMP-2 in the repair of alveolar clefts in humans (25). When Bouxsein and coworkers (26) assessed the retention time of ^{125}I-rhBMP-2 in absorbable collagen sponge using gamma scintigraphy, they showed that approximately 37(+/-10)% of the initial dose remained at the site one week after surgery and 8(+/-7)% remained after two weeks. It is thus possible that genistin could be retained by collagen matrix carrier in a similar way and be released over time to exert its effect and lead to an increase in bone formation. Our study has considerable implications, as genistin is a common health supplement used for its multiple health maintenance effects. This is the first study to demonstrate its significant local osteogenic effect.

6. CONCLUSION

Genistin in collagen matrix is effective at increasing new bone formation locally, and can be used for bone grafting or for bone induction. Further research is necessary to optimize its use and to gain further understanding of the mechanisms of its impact on bone formation.

7. ACKNOWLEDGMENTS

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Abbreviations: ERs: estrogen receptors; BMP: bone morphogenetic protein; ANOVA: one-way analysis of variance; SD: standard deviation

Key Words: Bone Repair, Bone Graft, Genistin

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