1. ABSTRACT

The amount of new bone formed in collagen matrix with daidzein was compared to that formed in collagen matrix alone. Eighteen bone defects, 5mm by 10mm were created in parietal bone of 9 New Zealand white rabbits. In the experimental group, 6 defects were grafted with collagen matrix with daidzein. In the control groups, 6 defects were grafted with collagen matrix alone (positive control) and 6 were left empty (negative 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 100 sections (50 sections for each group of daidzein and positive control) using image analysis. A total of 602% more new bone was present in defects grafted with daidzein in collagen matrix than those grafted with the collagen matrix alone. Very little new bone formed in the negative control group. In conclusion, daidzein in collagen matrix has the effect of increasing new bone formation locally and can be used for bone grafting.

2. INTRODUCTION

Over the years autogenous cancellous bone grafting has been considered the gold standard in replacing bone (1). The major limitations of using autogenous grafting are the inadequacy of supply and surgical morbidity; including donor site pain, paresthesia, and infection, which can approach 8% to 10% (2). Moreover, graft resorption posed a severe problem. In an experimental study, endochondral bone grafts showed 65% volume loss (3). Allografts, an alternative to autogenous grafting, seem to be biologically inferior and are associated with infection and inflammation (4).

Therefore, discovery of chemicals that increase bone formation is needed to improve bone healing after bone grafting surgery. The decrease in serum estrogen after menopause is associated with bone loss and osteoporosis, and estrogen replacement therapy is considered to be effective in preventing bone loss (5). It has been shown that estrogen enhances osteoblast differentiation and bone formation (6) and the conditioned medium of estrogen-
Daidzein and bone

![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.](image)

Figure 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.

Phytoestrogens are plant-derived non-steroidal compounds that bind to ERs and have estrogen-like activity (8). Phytoestrogens have attracted much attention among public and medical communities because of their potential beneficial role in prevention and treatment of cardiovascular diseases, osteoporosis, diabetes and obesity, menopausal symptoms, renal diseases and various cancers (9-10).

Phytoestrogens are divided into three classes: isoflavones, coumestans and lignans. In addition, some flavonoids, such as flavonols, are also classified as phytoestrogens (10). Estrogen receptors exist as two subtypes, ERa and ERb, and osteoblasts express both receptors (11). The interaction between flavonols and flavonoids with ERa and ERb is well documented (12).

Daidzein belongs to the isoflavone class of flavonoids. Daidzein has been found to have both weak estrogenic and weak anti-estrogenic effects. Daidzein is mainly found in legumes, such as soybeans and chickpeas and is found in high quantities in soy foods (13). In Asia, population groups who eat soy foods daily have lower rates of breast and prostate cancer than do groups in the West (13).

BMP-2, a potent inducer of osteogenic differentiation, is a target for isoflavones (14). Daidzein increased the expression of mRNA encoding BMP-2 in primary rat osteoblastic cells (1).

As daidzein is present in large amounts in everyday Asian diets, if it 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 the present study we examined 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, we measured the amount of new bone produced by daidzein with collagen matrix carrier grafted into bony defects in rabbits and compared with that of the collagen carrier alone.

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 formed in collagen matrix carrier with daidzein grafted into bony defects and compared with that of the collagen carrier alone.

3. MATERIALS AND METHODS

3.1. Experimental and control groups

Eighteen 10×5mm² full-thickness bone defects were created in the parietal bones of 9 New Zealand White rabbits from an inbred colony. The rabbits were 5 months old (adult stage) and weighed 3.5-4.0kg. 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, 6 defects were grafted with collagen matrix carrier with daidzein. In the control groups, 6 defects were grafted with collagen matrix alone (positive control) and 6 were left empty (negative control). For the daidzein 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. This was performed so that the results can be compared with similar studies (15-16).

3.2. Surgical Procedures

The details of the operation and the postoperative care of the animals were previously described (15). In short, the animals were premedicated 1 hour before surgery with oxetetraacycline hydrochloride (200mg/mL, 30mg/kg body weight, Tetroxyla, Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3mL/kg body weight, Hypnorm, Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5mg/mL, 1mg/kg body weight, Valium 10, Roche). In order to maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1mL/kg) were given at 30-min intervals during the operation.

The surgical procedure consisted of the creation of two 10mm by 5mm full-thickness (approximately 2mm) cranial defects, devoid of peristomeum, using templates, in the parietal bones (Figure 1). The defects were produced using round stainless steel burs (1mm in diameter) on a low speed dental drill. Outlines of the defects were made.
Daidzein and bone

Figure 2. Photomicrograph of bony defect grafted with daidzein 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 ×40).

Figure 3. Photomicrograph of bony defect grafted with collagen matrix (positive control) in 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 ×40).

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 daidzein group, the defects were filled with collagen matrix with daidzein consisted of 0.2mL daidzein solution (Sigma-Aldrich, MO, USA, dissolved in water for injection to the concentration of 100mg/mL, this dose was determined by pervious pilot study), mixed with 0.02g of collagen matrix (purified absorbable fibrillar collagen, Collagen Matrix Inc, NJ, USA). The grafts were prepared 15 minutes before grafting. In the control groups, the defects were either left untreated (negative control) or grafted with 0.02g of collagen matrix (purified fibrillar collagen, Collagen Matrix, Inc NJ, USA) mixed with 0.2mL water for injection (positive control).

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 with sodium pentobarbitone. Immediately upon death, defects and surrounding tissue were removed for histological preparation.

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 finally double embedded in celloidin/ paraffin wax. Serial, 5-μm-thick sections of the whole defect were cut perpendicular to the long axis. The slides were stained with Periodic acid-Schiff stain which allowed easy identification of new bone.

Quantitative analysis was made on serial sections of defects in the experimental and the active control groups. Defects were divided into 5 regions spaced 1500μm apart (Figure 1). From more than 10 sections in each region, 2 sections were selected randomly, giving a total of 10 sections from each defect. Therefore, the amount of new bone formation was assessed throughout the whole defect. The total amount of new bone formed (N, Figure 2-3) within the surgically-created defect was measured on 100 sections with a technique previously described (15-16).

Each section included the graft and host bone of both sides of the defect. Thus there were 2 graft-host interfaces. The total amount of new bone (N, Figure 2-3) formed in both graft-host interfaces within the surgically created defect was quantified by one observer (blinded) who did not know which group he was measuring by outlining the periphery of the newly formed bone. (Leica Qwin Image Processing & Analysis Software, V2.3, Leica Microsystems Imaging Solutions Ltd, UK) through a transmitted light microscope (Leica, DMLB, Germany) fitted with a video camera (Single CCD Color Camera, Tk-C1380E, JVC, Japan). With this software system, the periphery of the new bone (N, Figure 2-3) was outlined on the computer screen using the mouse manually and the area of the outlined bone could then be calculated by the software. Differences in staining properties and morphology between newly-formed bone and mature bone made identification easy (Figure 2-3).

3.5. Statistical Methods

Data were analyzed using a statistical analysis computer software (Graphpad Instat, v.2.04a, 1993, San Diego, CA, USA). The ANOVA method was used to compare sections drawn from the five regions in each defect. The arithmetic mean, SD and 95 per cent confidence intervals were calculated for each experimental group. The means (daidzein group and control group) were compared by the 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 outlining the areas of new bone was calculated by the formula where method error equals to the square root of (the sum of the

3675
Daidzein and bone

Figure 4. High power photomicrographs showing the formation of new bone in a bony defect grafted with daidzein in collagen matrix. Box in the left photomicrograph was enlarged and shown on the Figure 5. New bone (N) near the host bone (H) could be seen growing around and towards the collagen matrix (C). (Periodic acid-Schiff stain, original magnifications: ×80).

Figure 5. High power photomicrographs showing the formation of new bone in a bony defect grafted with daidzein in collagen matrix. Osteocytes (O) could be seen growing around and towards the collagen matrix (C). Capillaries (cap) were present. No cartilage was found. (Periodic acid-Schiff stain, original magnifications: ×400).

squared differences between the two sets of two mean values over the double of the number of double measurements). Ten randomly drawn histological sections were outlined on two separate occasions at least three months apart by the same observer and also by an independent observer. Paired t-tests were also performed to compare the intra-observer and the inter-observer registrations. The two-tailed P value to compare the intra-observer registrations was 0.5652, that to compare the inter-observer registrations was 0.5911, both 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 after operation. There was no evidence of side effects or infection in any of the animals.

4.2. Histological findings

Daidzein group: In the group grafted with daidzein in collagen matrix, new bone was formed at the host bone – graft interface and tended to grow across the defect (Figure 2). Integration of the daidzein and collagen with the recipient bed was characterized by the presence of new bone. No cartilage was found. At higher magnification (Figure 4-5), new bone could be seen growing around a collagen matrix fragment and tended to growth towards and amalgamate with the collagen matrix, bone cells were present showing that the collagen was not just calcified, rather, new bone was formed.

Control groups: In the active control group little new bone was formed at the host bone – graft interface. Some collagen fibers were present at the centre of the defects (Figure 3, 7). In the passive control group, the defect was healed, with fibrous tissue bridging across the defect. 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 100 sections of the daidzein group and the active control group were digitized and analyzed.

The amount of newly formed bone was significantly greater in the defects grafted with daidzein in collagen matrix than in those grafted with collagen matrix only (Tables 1 - 2), (Figure 6). In the daidzein group, 50 sections were measured; the mean area of newly formed bone in each defect was 2.88mm², with a standard deviation of 0.7872mm². In the positive control group, 50 sections were measured; the mean area of newly formed bone in each defect was 0.41mm², with a standard deviation of 0.2728mm². Unpaired t test with Welch correction which does not assume equal variances was used to test the difference between the two groups, the two-tailed P value is <0.0001, considered significant.

5. DISCUSSION

Our laboratory demonstrated that daidzein in collagen matrix significantly enhanced new bone formation locally when grafted into skull defects. It produced 602% more new bone than the collagen matrix alone (positive control), (Figure 2-6). The difference was significant (P<0.0001, unpaired t test). This comparison showed that daidzein increased bone formation when used with collagen matrix. Compared with similar studies daidzein was one of the strongest osteogenic chemical ever discovered (16-20).

The sample size was based on previous research using this model (15-20). Our laboratory used a small sample size because there was a large difference in bone formation between different groups in pilot study so that statistical significant difference can be detected with minimal number of animals, also required by the animal ethic committee that the smallest sample size that allows a significant difference to be detected should be used.
Table 1. Comparison of amounts of new bone (mm²) in five defects grafted with daidzein in collagen matrix

<table>
<thead>
<tr>
<th>Region</th>
<th>Defect 1</th>
<th>Defect 2</th>
<th>Defect 3</th>
<th>Defect 4</th>
<th>Defect 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.06</td>
<td>2.52</td>
<td>2.46</td>
<td>3.12</td>
<td>3.91</td>
</tr>
<tr>
<td>B</td>
<td>2.11</td>
<td>2.35</td>
<td>2.64</td>
<td>3.44</td>
<td>3.91</td>
</tr>
<tr>
<td></td>
<td>1.69</td>
<td>2.94</td>
<td>2.53</td>
<td>4.13</td>
<td>3.66</td>
</tr>
<tr>
<td>C</td>
<td>2.23</td>
<td>3.67</td>
<td>2.76</td>
<td>4.44</td>
<td>3.43</td>
</tr>
<tr>
<td>D</td>
<td>2.14</td>
<td>4.07</td>
<td>2.60</td>
<td>4.41</td>
<td>4.12</td>
</tr>
<tr>
<td></td>
<td>2.29</td>
<td>2.74</td>
<td>2.07</td>
<td>3.48</td>
<td>2.76</td>
</tr>
<tr>
<td></td>
<td>2.37</td>
<td>2.79</td>
<td>1.85</td>
<td>3.65</td>
<td>2.88</td>
</tr>
<tr>
<td>E</td>
<td>1.93</td>
<td>2.16</td>
<td>1.79</td>
<td>2.83</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td>2.26</td>
<td>2.22</td>
<td>1.94</td>
<td>2.69</td>
<td>2.84</td>
</tr>
<tr>
<td>Mean</td>
<td>2.113</td>
<td>2.84</td>
<td>2.317</td>
<td>3.623</td>
<td>3.5</td>
</tr>
<tr>
<td>SD</td>
<td>0.1979</td>
<td>0.6168</td>
<td>0.3637</td>
<td>0.6258</td>
<td>0.6182</td>
</tr>
<tr>
<td>df</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>F</td>
<td>2.200</td>
<td>42.642</td>
<td>21.846</td>
<td>53.860</td>
<td>8.135</td>
</tr>
<tr>
<td>P</td>
<td>0.2050</td>
<td>0.0005</td>
<td>0.0023</td>
<td>0.0003</td>
<td>0.0205</td>
</tr>
</tbody>
</table>

Table 2. Comparison of amounts of new bone (mm²) in five defects grafted with collagen matrix (positive control)

<table>
<thead>
<tr>
<th>Region</th>
<th>Defect 1</th>
<th>Defect 2</th>
<th>Defect 3</th>
<th>Defect 4</th>
<th>Defect 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.68</td>
<td>0.64</td>
<td>0.18</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>B</td>
<td>0.74</td>
<td>0.82</td>
<td>0.18</td>
<td>0.11</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.57</td>
<td>0.68</td>
<td>0.15</td>
<td>0.31</td>
<td>0.29</td>
</tr>
<tr>
<td>C</td>
<td>0.51</td>
<td>0.71</td>
<td>0.16</td>
<td>0.38</td>
<td>0.34</td>
</tr>
<tr>
<td>D</td>
<td>0.70</td>
<td>0.85</td>
<td>0.35</td>
<td>0.23</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>0.96</td>
<td>0.32</td>
<td>0.18</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
<td>0.86</td>
<td>0.20</td>
<td>0.22</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>0.74</td>
<td>0.95</td>
<td>0.21</td>
<td>0.24</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.51</td>
<td>0.06</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.80</td>
<td>0.62</td>
<td>0.06</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>Mean</td>
<td>0.662</td>
<td>0.76</td>
<td>0.187</td>
<td>0.145</td>
<td>0.247</td>
</tr>
<tr>
<td>SD</td>
<td>0.09223</td>
<td>0.1503</td>
<td>0.09393</td>
<td>0.07412</td>
<td>0.1431</td>
</tr>
<tr>
<td>df</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>F</td>
<td>1.379</td>
<td>6.494</td>
<td>179.23</td>
<td>34.071</td>
<td>96.734</td>
</tr>
<tr>
<td>P</td>
<td>0.3603</td>
<td>0.0324</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

The rabbit model used in this study was relevant because non-grafted control bone defects have been found not to heal with new bone formation within fourteen days after their creation. In addition, there was minimal bone healing across the defect with collagen matrix as shown by the results of the control group (Figure 3, 6). There was minimal morbidity due to this procedure as all the rabbits were in good health and condition after the surgery. The bone formation was examined after fourteen days, this time span allowed investigation of the ability of new bone to grow across the bone defect during the early healing phase. This time span had also been used in other studies on bone formation.
Daidzein and bone

Figure 7. Comparison of areas (mm²) of newly formed bone between defects grafted with collagen matrix (A, 2.88, SD=0.7872), and defects grafted with daidzein in collagen matrix (B, 0.41, SD=0.2728).

formation using the same animal model (15-20), therefore comparisons between studies can be made.

The results of the ANOVA of the different regions within each defect showed the necessity to analyze multiple regions within each defect (Tables 1-2). This was indicated by the P value, the difference in the amount of new bone formed between the different regions within each defect was statistically significant in many defects. Despite the variations in the amount of bone formation in different regions and in different defects, the amount of bone formation of the daidzein group was greater than that in the control groups.

Collagen matrix (purified absorbable fibrillar collagen) was used in this study as a carrier. It was derived from bovine tendon, in the fibrillar form and was suggested from the Manufacturer (Collagen Matrix, Inc NJ, USA) to be useful for delivering cells and growth factors and for gene therapy. It had been used successfully as a carrier for growth factors like BMP-2 in bone research (21-23). Recently it was successfully used with rhBMP-2 in the repair of alveolar clefts in humans (24). Bouxsein and coworkers (25) assessed the retention time of 125IrhBMP–2 in absorbable collagen sponge using gamma scintigraphy and showed that about 37(±10)% of the initial dose remained at the site one week after surgery, and 8(±7)% remained after two weeks. It is possible that daidzein could have been retained similarly by the collagen matrix carrier and released over time to exert their effect that led to an increase in bone formation. However, a direct comparison between BMP and daidzein cannot be made because BMP is a protein and daidzein is not.

The dose for daidzein in this study was estimated from the dose used in an other study with phytoestrogen. In pilot study this estimated dose has caused an increase in bone formation. In this study we have tried to demonstrate the increased bone formation effect of daidzein using one commonly used carrier for bone repair. Further research is needed to determine the optimum dose, best choice of carrier and the release kinetics of daidzein for bone grafting.

This study has considerable significance as daidzein is a commonly taken natural compound. This might be among the first studies that demonstrated its local effect in increasing bone formation. Daidzein has potential to be utilized in any application that requires increase in bone formation, especially in patients that have compromised host responses.

6. CONCLUSION

Daidzein in collagen matrix have the effect of increasing new bone formation locally and can be used for bone grafting. Further research is needed to optimize its use and to gain further understanding on its bone increasing mechanism.

7. ACKNOWLEDGEMENTS

This work was supported by the University Research Grant no.: 10207357/36170/08003/323/01, the University of Hong Kong.

8. REFERENCES

Daidzein and bone


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

**Key Words:** Bone Repair, Bone Graft, Daidzein

**Send correspondence to:** Ricky W. K. Wong, 2/F, Orthodontics, Prince Philip Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong, Tel: 852-28590554, Fax: 852-25593803, E-mail: fyoung@hkucc.hku.hk