Effect of Flaxseed Application on Bone Healing in Male Rats, Histological and Immunohistochemical Evaluation of Vascular Endothelial Growth Factor

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Background and Objectives: Bone repair is a multistep process that involves migration, proliferation, differentiation and the activation of several cell types. Bone is a highly vascularised tissue that is reliant on the close spatial and temporal connection between the blood vessels and bone cells to maintain skeletal integrity. Therefore, angiogenesis plays a pivotal role in skeletal development and bone fracture repair. In this study, the role of angiogenic and osteogenic factors in the adaptive response and interaction of osteoblasts and endothelial cells during the multistep process of bone development and repair have been highlighted. Furthermore, the role of the local exogenous application of flaxseed in bone healing was identified and the immunohistochemical expression of VEGF in a rat model of bone defects was analyzed.

Materials and Methods: Twenty-one male rats, weighing 350-400 g, were subjected to a surgical operation of the medial sides of both tibiae bones (the right side was considered as the experimental site and the left side as the control), in the control, the bone defect was treated with a local application of 1 μL normal saline, whereas, the experimental site was treated with a local application of 1 μL flaxseed oil. Seven rats were killed at 3, 7 and 14 days after surgery and bone healing was histologically examined through the immunohistochemical localization of VEGF. All statistical analyses were computed using GraphPad Prism version-5.

Results: Bone defects treated with the local application of flaxseed showed early osteoid tissue deposition with high cell counts of osteoblasts, osteocytes and osteoclasts. Immunohistochemical staining for VEGF in stromal cells revealed a higher expression in the flaxseed group in comparison with the control. Conclusion: The low dose application of flaxseed could be an effective therapeutic treatment for bone injuries. These data indicate the possibility of future clinical applications for flaxseed.

Key words: Vascular endothelial growth factor, flaxseed, bone healing, osteogenic factors, immunohistochemistry
INTRODUCTION

Bones are responsible for mechanical supporting the soft tissues and muscles as well as its essential roles in calcium homeostasis. Bone healing is similar to that of soft tissue healing but without formation of scar tissue. Bone healing involves a consequent events starting with haematoma formation, inflammatory reaction, cartilaginous formation, vascularization, mineralization and hard callus formation which will remodelled by osteoclasts activity. Bones are responsible for mechanical supporting the soft tissues and muscles as well as its essential roles in calcium homeostasis. Bone healing is similar to that of soft tissue healing but without formation of scar tissue. Bone healing involves a consequent events starting with haematoma formation, inflammatory reaction, cartilaginous formation, vascularization, mineralization and hard callus formation which will remodelled by osteoclasts activity. Although bone tissue usually heals spontaneously but in difficult conditions a numerous therapeutic modalities and supplements are needed to enhance the healing response. Different types of growth factors have beneficial role for treatment and achievement a successful bone healing.

The goal of bone healing is to regenerate mineralized tissue in the site of fracture. Various factors that enhance bone healing are types of biomaterial or graft used to rebuild the injured area and type of therapeutic agents that improve the response of bone healing. In histological terms, bone healing has been categorized into primary (direct) and secondary (indirect) steps. In Primary step, fixation methods that provide compression across the fracture let direct healing, where the osteoclasts in the tips of the cutting edges produce longitudinal cavities that will be filled with a regenerative bone by osteoblasts and the formation of Haversian canals followed by penetration of blood vessels and direct remodeling into lamellar bone and bone healing will be occur without hard callus formation. Secondary bone healing involves both intramembranous and endochondral ossification with hard callus formation. Secondary bone healing occur in three phases, including inflammatory, fibroplasia and repair with remodeling.

Comprehensive information of bone healing is by improving treatment and management of bone defects. Therefore, development of supportive materials to enhance bone healing based on osteogenic cells and osteoinductive factors could be considered as potential ways to create substitutes for biologic tissues in order to reconstruct bone defects.

One of the most challenges in clinical orthopaedic is the consequence of bone defect repairing, since the development of microvasculature and microcirculation, is critical for the homeostasis and regeneration of living bone, without this, the tissue would degenerate and die. Bone tissue has been shown to contain various intracellular signalling peptides called growth factors, which are thought to exert important regulatory functions, especially in bone remodelling and healing, based on their potent impact on osteocyte metabolism. In vivo studies have demonstrated that growth factors are candidates for future clinical use in orthopaedic surgery. In numerous clinical situations, enhanced bone formation and bone healing can lead to an improvement in the results of surgical procedures. Various factors, such as hormones, growth factors, different cytokines, chemokines and metabolites are present in the vasculature inside the bone tissue or surrounding tissues. These factors act as a limited barrier to the movement of molecules and cells. Various signals and attracting factors have been shown to be expressed on the endothelium of the bone this expression was found to be helpful for the recruitment of circulating cells, especially haematopoietic cells, into the bone marrow. These signals or factors may be coordinated with metastatic cells to direct them to the skeleton.

Flaxseeds also known as linseeds, are a rich source of dietary fibres and micronutrients, as well as several minerals, including manganese, vitamins (particularly the vitamin B complex) and the essential fatty acids (particularly linolenic acid, which is also known as omega-3). The seeds are obtained from flax, which is considered one of the oldest fibre crops in the world and was cultivated in ancient Egypt and China. Flaxseed is a good source of omega-3, antioxidants and fibres and has been identified to possess hypoglycaemic, anticancer and cardioprotective activity. Many studies have reported the protective effects of flaxseed against prostate, colon and breast cancer. These therapeutic effects may be attributable to its ability to prevent the growth of cancerous cells through its content of omega-3 fatty acid, which was shown to the adherence of disrupt malignant cells to other body cells. A flaxseed containing diet may protect skin from radiation damage. Flaxseed, known for its strong antioxidant and anti-inflammatory properties, functions as both a mitigator and protector against radiation pneumonopathy.

For assessment of bone healing in experimental animals, immunohistochemical studies are helpful methods to give mechanistic data and help the experimental results to be discussed. Collagen types I, as an indicator of osteogenic activity and VEGF, as an important contributor for angiogenesis, consequent resorption of cartilage by chondroclasts and some non-collagenous proteins such as osteopontin can be assessed to evaluate bone healing.

The objective of the research was to study the benefits of the local application of flaxseed oil as an exogenous biomaterial on bone healing. This is the first study to test the use of flaxseed oil in the bone healing process.

MATERIALS AND METHODS

Experimental animals: The present study was conducted at the College of Dentistry, University of Baghdad, from October
Materials

Flaxseed oil (Vintage Pharmaceuticals, LLC): Polyclonal antibodies of Vascular Endothelial Growth Factor Antibody (VEGF) from Abcam, UK (ab46154).

Experimental groups: The animals were subjected to a surgery, which was performed under sterilized conditions using a gentle technique. Each animal was weighed to calculate the appropriate dose of general anaesthetic. General anaesthesia was induced via intramuscular injection of xylazine 2% (0.4 mg kg\(^{-1}\) b.wt.) and ketamine HCl 50 mg (40 mg kg\(^{-1}\) b.wt.) and an antibiotic of oxytetracycline 20% (0.7 mL kg\(^{-1}\) b.wt.) was also administered via intramuscular injection. An incision was performed on the lateral side to expose the medial side of the tibia and the skin and fascia flap was reflected. During the instrument drilling and continuous cooling with irrigated saline, a 2 mm hole was made with a small round bur with a rotary speed of 1600 rpm. After the whole process, the operation site was washed with saline solution to remove debris from the drilling site. The bone defect was made on both the medial sides of right and left tibia bone and classified as experimental and control sites, respectively. After the operation, the area was air dried, then 1 μL flaxseed oil was applied to the experimental site and 1 μL normal saline was applied to the control site. The muscles were sutured with absorbable catgut, which was followed by skin suture. The operation site was sprayed with a local antibiotic. The animals were killed after 3, 7 and 14 days by using an overdose of anaesthesia. Seven animals were analysed for each period in each group, the bones were removed and the tibial bone was dissected and fixed in 10% buffered formalin.

Methods: Histological (H and E staining) and immunohistochemical evaluation were performed in accordance with Abcam company instructions. In the immunohistochemical analysis, positive readings were indicated by a brown cytoplasmic stain, whereas, negative readings were indicated by the absence of immune reactions in the negative control. Immunohistochemical scoring was conducted for VEGF-positive cells. The immunoreactivity was semi-quantitatively estimated based on the immunostaining score, which was calculated from the sum of a proportion score and an intensity score. The proportion score reflected the estimated fraction of positively stained infiltrating cells (score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; score 4, >80%)\(^2\).

Statistical analysis: The values for bone cell count and stromal cells expressing VEGF are expressed as the Mean±SD. The comparisons between the control and treated groups were performed using Student’s t-test. The comparisons between the periods were performed using the F-test. Differences were considered significant if values of p<0.05 were obtained. All statistical analyses were computed using GraphPad Prism version-5 (GraphPad Software, Inc. California, USA).

RESULTS

Histological findings

Control group: Three days after the induction of the bone defect, inflammatory and fibroblast cells were observed (Fig. 1a). After 7 days, the bone defect showed bone deposition of the collagen matrix and collagen bundles (Fig. 1b). After 14 days, sparse bone trabeculae were observed (Fig. 1c).

Experimental group (treated with flaxseed): The bone defect was treated with flaxseed. After a treatment for 3 days, the deposition of osteoid tissue with inflammatory cells was observed (Fig. 2a). After 7 days, trabeculated bone (TB), coalesce with cutting bone (CB) and bone trabeculae deposit were observed in the bone defect (Fig. 2b). After 14 days, the bone defect showed new bone (NB) with multiple haversian canals (HC) and nearly cutting bone (CB) (Fig. 2c).

Immunohistochemical findings: After 3 days, the immunohistochemical analysis revealed positive expression of VEGF in progenitor cells and endothelial cells (Fig. 3a), which was also present after 7 days (Fig. 3b). After 10 days, the fibroblast cells and active osteocyte cells showed positive VEGF expression (Fig. 3c).

In the flaxseed treated group, positive VEGF expression in progenitor cells and fibroblast cells was observed after 3 days (Fig. 4a). After 7 days, the osteoblasts and osteocytes, progenitor cells and endothelial cells showed positive VEGF expression (Fig. 4b and c). After 14 days, osteoclasts showed positive VEGF expression, in addition to osteoblasts and osteocytes (Fig. 4d and e).

The descriptive statistical analysis of bone cell counts is provided in Table 1 and revealed a significant (p<0.05) increase in osteoclasts, osteocytes and osteoclasts after 14 days of flaxseed treatment in comparison with the control group, however, decreased counts were shown after 28 days of treatment. The positive stromal cell count in the flaxseed-treated animals increased (p<0.05) after 3, 7 and 14 days of treatment with flaxseed in comparison with the corresponding periods in the control group (Table 2).
Fig. 1(a-c): Histopathological evaluation of the bone defect in the control group, (a) Microphotograph of the bone defect in the control group shows the presence of inflammatory cells (arrow) after 3 days, H and E ×10, (b) Collagen bundles (arrow) filled the bone defect of control group after 7 days, H and E ×20 and (c) Sparse bone trabeculae were observed during the healing of the bone defect in the control group after 14 days, H and E ×10

Fig. 2(a-c): Histopathological evaluation of the bone defect in the flaxseed treated group, (a) Deposition of osteoid tissue (OST) with inflammatory cells (arrow) in the bone defects treated with flaxseed was observed after 7 days, H and E ×10, (b) Trabeculated bone (TB) and coalesce with cutting bone (CB) were observed in the flaxseed treated group after 7 days, H and E ×20 and (c) New bone (NB) with multiple haversian canal (HC) and nearly cutting bone (CB) were observed after 7 days, H and E ×20
Fig. 3(a-c): (a) Immunohistochemical analysis of the control group revealed positive expression for VEGF in progenitor cells (arrow heads) and endothelial cells (arrows), DAB stain ×40, (b) Progenitor cells (arrow heads) and endothelial cell (arrows) showed positive VEGF expression in the control group after 7 days, DAB stain ×20 and (c) A fibroblast (arrow) and active osteocytes (arrow heads) show positive VEGF expression in the control group after 14 days, DAB stain ×40

Table 1: Descriptive statistical analyses of the bone cell counts (osteoblasts and osteoclasts) in the experimental groups (control and flaxseed-treated) after 7, 14 and 28 days, as determined by H and E staining

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration (days)</th>
<th>H and E bone cells count</th>
<th>No.</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>Lower bound</th>
<th>Upper bound</th>
<th>Max.</th>
<th>Min.</th>
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<tr>
<td>Control - Osteoblast</td>
<td>Days 7</td>
<td>7</td>
<td>7</td>
<td>8.67</td>
<td>1.03</td>
<td>0.42</td>
<td>7.58</td>
<td>9.75</td>
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<td>10</td>
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<td></td>
<td>Days 14</td>
<td>7</td>
<td>7</td>
<td>6.00</td>
<td>1.10</td>
<td>0.45</td>
<td>4.85</td>
<td>7.15</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Control - Osteocyte</td>
<td>Days 7</td>
<td>7</td>
<td>7</td>
<td>10.67</td>
<td>3.88</td>
<td>1.58</td>
<td>6.59</td>
<td>14.74</td>
<td>7</td>
<td>18</td>
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<tr>
<td></td>
<td>Days 14</td>
<td>7</td>
<td>7</td>
<td>8.67</td>
<td>2.07</td>
<td>0.84</td>
<td>6.50</td>
<td>10.83</td>
<td>6</td>
<td>12</td>
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<tr>
<td>Control - Osteoclast</td>
<td>Days 7</td>
<td>7</td>
<td>7</td>
<td>1.00</td>
<td>0.63</td>
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<td>0.34</td>
<td>1.66</td>
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<td>2</td>
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<tr>
<td></td>
<td>Days 14</td>
<td>7</td>
<td>7</td>
<td>0.50</td>
<td>0.55</td>
<td>0.22</td>
<td>-</td>
<td>1.07</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Paste - Osteoblast</td>
<td>Days 14</td>
<td>6</td>
<td>6</td>
<td>17.33</td>
<td>2.66</td>
<td>1.09</td>
<td>14.54</td>
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<td>20</td>
</tr>
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<td></td>
<td>Days 28</td>
<td>6</td>
<td>6</td>
<td>6.83</td>
<td>0.98</td>
<td>0.40</td>
<td>5.80</td>
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<tr>
<td>Paste - Osteocyte</td>
<td>Days 14</td>
<td>6</td>
<td>6</td>
<td>14.17</td>
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<td>Days 28</td>
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<td>6</td>
<td>13.00</td>
<td>0.89</td>
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<td>12.06</td>
<td>13.94</td>
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<td>14</td>
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<td>Paste - Osteoclast</td>
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<td>6</td>
<td>6</td>
<td>1.83</td>
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<td>0.31</td>
<td>1.04</td>
<td>2.62</td>
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<td>3</td>
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<tr>
<td></td>
<td>Days 28</td>
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<td>6</td>
<td>0.17</td>
<td>0.41</td>
<td>0.17</td>
<td>-</td>
<td>0.60</td>
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Table 2: Descriptive statistical analyses of positive stromal cells, as expressed by the VEGF marker, in the experimental groups (control and flaxseed-treated) at 3, 7 and 14 days (H and E)

<table>
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<th>Descriptive statistics</th>
<th>Duration difference</th>
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<td></td>
<td></td>
<td>Mean</td>
<td>S.D.</td>
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<td>41.50</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>28.13</td>
<td>0.85</td>
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<td></td>
<td>14</td>
<td>11.43</td>
<td>0.33</td>
</tr>
<tr>
<td>Flaxseed oil</td>
<td>3</td>
<td>63.28</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>50.13</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>37.60</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Fig. 4(a-e): (a) Immunohistochemical analysis of the flaxseed treated group showed positive staining for VEGF in the progenitor cells (arrow heads) and fibroblasts (arrows), DAB stain ×40, (b) Osteoblast (arrows) and osteocytes (arrow heads) show positive VEGF expression in the flaxseed group after 7 days, DAB stain ×40, (c) Progenitor cells (arrow heads) and endothelial cell (arrows) show positive VEGF expression, DAB stain ×40, (d) An osteoblast (arrow) and osteocytes (arrow heads) show positive VEGF expression in the flaxseed-treated group after 14 days, DAB stain ×40 and (e) An osteoclast cell (arrow) with positive VEGF expression, DAB stain ×40

DISCUSSION

Bone formation, also called ossification, is the process by which new bone is produced. Ossification begins at approximately the 3rd month of foetal life in humans and is completed by late adolescence. To elucidate the extent to which skeletal regeneration was affected by the exogenous application of flaxseed oil, a detailed analysis of the stages of bone apposition and ossification was conducted. The present study showed early osteoid deposition in the flaxseed treated group after 3 days, in the form of osteoid tissue formation, accompanied by significant immunopositive expression of VEGF in bone defect sites. These results could be attributed to activity at the traumatic site, including the differentiation of stem cells into osteoblasts, that was enhanced by flaxseed, which has been previously implicated in angiogenesis and proliferative osteoblasts and active osteocytes that were included in the deposition of collagen fibre.

After 7 and 14 days, the histological analysis revealed that the flaxseed treated groups showed trabeculae that filled approximately the whole defect in comparison with the control group. This result was attributable to the direct induction of primary osteoblast differentiation by flaxseed, which is responsible for bone apposition and mineralization. The control treated defects were not able to create a bony bridge across the gap. In contrast, flaxseed treatment caused significant filling of the defect with bone.
In rats, the direct exposure of the bone-forming cells, called osteoblasts, to these flaxseed compounds resulted in a significant increase in the rate of bone growth and bone strengthening within a few days. Some of this boosting effect is a result of the activity of a key enzyme that promotes bone growth.

The present study used exogenous flaxseed oil treatment in a bone defect owing to its content of short-chain omega-3 fatty acids, which are essential fatty acids (EFAs) and may offer certain health benefits. This is the first trial to investigate the use of flaxseed oil in the treatment of bone defects. Flaxseed oil has a very high content of alpha-linolenic acid [C18:3n3, omega-3 (n3) fatty acid]. Based on the properties of n3 fatty acids in fish oil, it is marketed as a health food.

The present findings could be attributed to the healing properties of flaxseed oil due to its super rich content of omega-3 oils, which are includes essential fatty acids (ALA, EPA and DHA) that important for health as well as the presence of linamarin which is a protective substances that have anti-inflammatory property. The presence findings agreed with the results reported by Simopoulos and Gogus and Smith, whom attributed their findings to the presence of omega-3 fatty acids, when they used flaxseed in the treatment of arthritis, osteoporosis and neurological disorders. Therefor, the present findings may be attributed to the high quantity of omega-3 fatty acids, where it has been shown that omega-3 fatty acids play a role in enhancement of osteoblasts activity and inhibition of osteoclasts activity. On the other hand the ratio between omega-6 and omega-3 fatty acids could be another factor to play an important role in bone health and mineral deposit.

The present result was in disagreement with that reported by Boulbaroud et al. because they used flaxseed oil in overiectomized rats, where the ovaries is considered as a rich source of estradiol which is necessary for bone health, however, flaxseed oil contains lignans, which have phytoestrogenic as well as its antioxidant properties.

Carano and Filvaroff reported that an intimate connection, from both physical and biochemical perspectives, has long been recognized between blood vessels and bone cells. Genetic, biochemical and pharmacological studies have identified and characterized the factors involved in the conversation between endothelial cells (EC) and osteoblasts (OB) during both bone formation and repair.

The statistical evaluation of the overall treatment period showed that the flaxseed group recorded the highest value mean for VEGF-positive staining. Therefore, the present study suggested that flaxseed initiated the formation of a blood clot through the biochemical activation, recruitment and differentiation of mesenchymal cells, which was followed by cellular activation and finally a cellular response that released the growth factors, including VEGF. As VEGF itself has no osteo-inductive capacity, the bone-forming cells were most likely recruited from pericytes, circulating cytokines and cells, the blood clot and the fractured bone ends. VEGF potentiates the actions of several cytokines and mediates the angiogenic actions of most growth factors. It could therefore complement other cytokines, such as basic fibroblast growth factor or bone morphogenetic proteins, to achieve enhancement of bone healing. Endogenous VEGF is secreted from endothelial cells, fibroblasts, osteoblasts and other cell types; its expression and the expression of its receptor, may be upregulated by flaxseed. The findings of this study constitute a promising demonstration of the enhanced formation of bone after the topical application of flaxseed.

CONCLUSION

A low-dose application of flaxseed was shown to be an effective therapy for bone injury. The study suggested that a 14 day period represented the active period of new bone formation and VEGF-expression was observed in different cells, including osteoblasts, osteocytes, fibroblasts and endothelial cells. These data illustrated the potential of flaxseed for use in future clinical applications.

SIGNIFICANCE STATEMENTS

The present study reveals the possible effect of the application of flaxseed in the therapy of bone injuries. This study will assist researchers in the discovery of medicinal plants that can be applied as an alternative source of medication. These data suggest the possible future clinical applications of flaxseed.

REFERENCES