Histomorphometric evaluation of the effects of local application of red clover oil (Trifolium pratense) on bone healing in rats

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Abstract:
Objective: Red clover oil (Trifolium pratense) that have isoflavones bunches which estrogen-like exercises and may establish an option in contrast to hormone substitution treatment. The present examination researched the impacts of Red clover oil on bone healing in rats by histomorphometrical study

Methods: Intra bony defect was done in right femur of thirty six males healthy albino rats. Then these rats were randomly divided into three groups (12 rats for each) one control and 2 experimental group. For control, the bony defect left for normal healing, experimental (S) group the defect treated with hemostatic absorbable gelatin sponge, and for experimental (RS) group the bony defect treated with 0.2 ml red clover oil and covered by haemostatic absorbable gelatin sponge. Six rats from each group were sacrificed at 2 and 4 weeks intervals. Histolomorphometric analysis was performed on H&E bone section of all the studied groups which include counting of bone cells (osteoblasts, osteocytes and osteoclasts), trabecular number, trabecular area and bone marrow space area

Results: Histomorphometrical results for bone cells revealed that combination group stimulated large number of osteoblasts and osteocytes than in sponge and control group. Number of new bone trabeculae, trabecular area and bone marrow space area showed high mean value in combination groups than others. Highly significant group difference was observed in all histomorphometric parameters in all duration.

Conclusion: Red clover oil stimulated large number of osteoblasts and osteoclasts that indicate increase bone remodeling especially at 2 weeks interval when compared with sponge and control group.

Key words: Histomorphometric, Red clover, Isoflavones, Bone healing.

Introduction:
Bone mending is a profoundly effective procedure that takes into account the scar less recovery and redesigning of imperfections identified with the treatment of injury, pathology, or inborn variations.
from the norm. Bone fix was a multistep procedure including relocation, expansion, separation, and initiation of a few cell types [1]. Bone redesigning requires the relations between numerous bone cells to revamp, protect, or direct bone quality or potentially mineral homeostasis in light of adjusting natural impacts. There were four discrete stages to this procedure: initiation, retention, inversion, and development with ingestion; that is occurring through osteoclasts and osteoblasts, correspondingly [2]. Bone imperfections attempt accommodating recuperating strategy through synchronized accompanying advancement of skeletal and vascular segments in a delicate cartilage callus setting. Among this setting bone restoration restates a few of the equivalent cell and sub-atomic systems that delivers the embryonic bone [3].

Red clover (Trifolium falsification) is a perpetual herb developing in all mild and subtropical zones the world over. In a few societies, it is utilized as customary prescription. Other than its daidzein and genistein content, red clover shows a high substance of methylized forerunners: biochanin A and formononetin [4, 5]. Isoflavonoids mixes present in red clover oil are the primary dynamic substances of "phytoestrogens". Epidemiological and clinical research has appeared constructive outcomes of isoflavone utilization over bone and the danger of building up a few osteoporosis [6, 7].

**Materials and Methods:** Thirty six males healthy albino rats, aged between (4 -5) months weight ranged between (250 -300 mg) were used in this experimental study. All rats were kept under supervision and nursing from the staff of the animal house of Biotechnology Research Center \ University of Al-Nahrain, Baghdad. All experimental procedures were done in accordance with the ethical approval of animal experimental of College of Dentistry, University of Baghdad. Intra bony defect of about 2mm in width and 3mm in depth was done in right femur of each rat [8]. Then rats were randomly divided into three groups (12 rats for each group) as follow:

1) Control group ( C ) (twelve rats): the bony defect left for spontaneous normal healing.
2) Experimental group (S) (twelve rats): the bony defect treated with hemostatic absorbable gelatin sponge.
3) Experimental group (RS) (twelve rats): the bony defect treated with 0.2 ml[9] red clover oil (Trifolium pratense) and covered by haemostatic absorbable gelatin sponge.

Then six rats from each group were sacrificed at the end of recommended periods(2 and 4 weeks). The right femur were dissected and the soft tissue was removed to expose the entire bone to be cut at 5 mm away of the defect sides. The bone specimens immediately stored in 10% freshly prepared formalin and left for 2 days for fixation. Bone decalcification was performed by using formic acid sodium citrate solution which was prepared freshly from 2 solution (125 cc formic acid 90 %,125 cc distilled water and 50 mg sodium citrate , 250 cc distilled water ) [10].

Then bone tissue dehydrated with alcohol and embedded in paraffin. Sections of 5μm were prepared in the usual fashion, and stained with hematoxylin and eosin. Histological examination was performed using light microscope. Histomorphometric assessment of bone cells (osteoblast, osteocyte and osteoclast), trabecular area, trabecular numbers and bone marrow space area was performed by software program (Image J. exe), which is image processing program developed at the National Institutes of Health [11]

**Results:**

**At two weeks duration:**

**A – Control group:** Histological view of bone defect revealed sparse of bone trabecular coalesce with cutting bone in control group of 2 weeks duration. Osteocytes in newly formed trabecular enclosing areas of marrow tissue and large number of osteocytes are embedded in bone, osteoblasts noticed at peripheries of the trabecular, reversal line observed separating between old and new bone, progenitor cells noticed also, figure(3)

**B-sponge group(S):** The histological examination of this group after 2 weeks duration illustrated the deposition of bone trabeculae that replace areas of bone defect, the osteoblasts are seen at
peripheries of the bone, osteocytes seen trapped in bone, and reversal line between old and new bone; figure (4).

c-Combined red clover oil and sponge group (RS) Histological examination of this group after two weeks revealed thick well developed bone trabeculae filled defect area. Osteoblasts present on periphery of new bone with numerous blood vessels and inflammatory cells inside bone marrow and osteocytes present inside bone matrix Figures (5).

Statistical analysis of histomorphometric findings

Table (1) showed descriptive statistics of trabecular area (BTA), trabecular number (TN) and bone marrow space area (BMA) in control, sponge and combination groups in both healing periods (2 and 4 weeks). The mean values for the BTA increased with time and they were higher in combination groups than those in other groups in 4 weeks duration. While the mean values for TN decreased with time and they were higher in combination groups than others in both duration and the highest mean value was noticed in combination group in 2 weeks duration. The bone marrow space area showed decrease in mean values with time and were less in combination group as compared other groups. Whereas the lowest mean value were recorded in combination (RS) group in 2 and 4 weeks.

Comparison differences among all studied groups at different healing periods for histomorphometric variables (BTA, TN and BMA) by using ANOVA test were shown in table(2). The result showed a highly significant difference between all studies groups in both durations two and four weeks for these three variables.

Figure(3): View of 2 weeks of Control group showed basal bone (BB) blood vessels (BV), inflammatory cells (IC) and osteocytes (OC). H&E X40

Figure(4): View of 2 weeks of Sponge group showed new bone trabeculae (BT), osteoclasts (OCL) and inflammatory cells (IC). H&E X40.
Figure (5): View of 2 weeks of RS group showed new bone filled by osteocytes (OC) and lined by osteoblasts (OB), numerous blood vessels (BV), inflammatory cells (IC), osteoclasts (OCL) and reversal line (RL). H&E X40

Figure (6): View of 4 weeks of Control group showed osteoblasts (OB) lined Haversian canal, osteocytes (OC) filled bone. H&E X40

Figure (7): View of 4 weeks of Sponge group showed regular arrangement of osteocytes (OC) around haversian canal (HC) and osteoblasts (OB). H&E X40
Figure (8): View of 4 weeks of RS group showed mature bone (osteon formation) by regular arranged osteocytes (OC) around haversian canal (HC) and Osteoblasts (OB) seen riming (HC). H&E X40

Table (1): Descriptive statistics of control and experimental groups at different healing periods for BTA, TN, BMMA.

| Duration | Control group | | | | | Sponge group | | | | | | | RS group | | | | |
|----------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
|          | N. O. | Mean | S.D. | S.R. | Min. | Max. | N. O. | Mean | S.D. | S.R. | Min. | Max. | N. O. | Mean | S.D. | S.R. | Min. | Max. |
| Bone trabecular area | 2 weeks | 8 | 0.10 | 0.014 | 0.0017 | 0.18 | 0.197 | 8 | 0.186 | 0.016 | 0.015 | 0.107 | 0.21 | 8 | 0.225 | 0.02 | 0.00 | 0.1 | 0.233 |
|          | 4 weeks | 8 | 0.24 | 0.023 | 0.0032 | 0.23 | 0.219 | 8 | 0.252 | 0.025 | 0.004 | 0.247 | 0.25 | 8 | 0.290 | 0.03 | 0.00 | 0.2 | 0.312 |
| Bone trabecular IU | 2 weeks | 8 | 9.46 | 0.224 | 0.007 | 8.5 | 9.0 | 8 | 9.35 | 0.221 | 0.006 | 8.9 | 9.7 | 8 | 10.3 | 0.14 | 0.00 | 9.5 | 10.7 |
|          | 4 weeks | 8 | 5.04 | 0.104 | 0.014 | 4.4 | 5.7 | 8 | 5.126 | 0.113 | 0.005 | 4.5 | 5.6 | 8 | 7.21 | 0.11 | 0.00 | 6.5 | 7.9 |
| Bone marrow area | 2 weeks | 8 | 0.65 | 0.032 | 0.0027 | 0.54 | 0.63 | 8 | 0.297 | 0.024 | 0.0018 | 0.29 | 0.31 | 8 | 0.269 | 0.02 | 0.00 | 0.1 | 0.29 |
|          | 4 weeks | 8 | 0.21 | 0.028 | 0.0023 | 0.18 | 0.29 | 8 | 0.163 | 0.012 | 0.0011 | 0.14 | 0.17 | 8 | 0.100 | 0.00 | 0.00 | 0.09 | 0.11 |


Table (3) illustrated the descriptive statistic of the number, mean, standard deviation, standard error, minimum and maximum values of bone cells measured at different healing periods (2 and 4 weeks) for the studied groups. The mean values of osteoblasts and osteocytes increased slightly with time for all studied groups.

On the other hands the highest mean value for both osteoclasts and osteocytes were recorded in combination group (RS) at four weeks duration. According to the ANOVA test, there was a highly significant difference among all studied groups in both 2 and 4 weeks intervals in bone cells except for osteoclasts at 4 weeks duration which was significant as shown in table (4).

Table (3) descriptive statistics of bone cells at different healing period for all groups

| Variable               | Duration | Group comparisons | F-test | p-value |
|------------------------|----------|-------------------|--------|---------|
| Bone trabecular area   | 2 weeks  | 333.5             | 0.0003** |
|                        | 4 weeks  | 429.9             | 0.0005** |
| Trabecular number      | 2 weeks  | 294.4             | 0.0002** |
|                        | 4 weeks  | 19.7              | 0.0001** |
| Bone marrow area       | 2 weeks  | 52.3              | 0.0006** |
|                        | 4 weeks  | 112.7             | 0.0008** |

* Significant difference (p < 0.05)  ** Highly significant difference (p < 0.01)
Discussion:
Within the last decade the use of natural supplements has become more widespread in the search for viable alternatives to existing treatments. Red clover (Trifolium pratense) is a medicinal herb containing flavonoids and isoflavones. Red clover contains at least 9 isoflavones including formononetin, biochanin A (glycosides), daidzein and genistein (aglycones) which promote the formation of bone [12,13]. Cellular processes stimulated include chemotaxis, mesenchymal cell proliferation and differentiation, angiogenesis, and synthesis of extracellular matrix. Although different isoflavones compounds are closely related structurally and functionally [14].

Portrayal of the recovered bone tissue is regularly performed by histological assessment with light microscopy, following standard recoloring of the example. Enlightening histology is utilized to give a general appraisal of the tissue of enthusiasm, giving information with respect to cell morphology,

Table (4) Group comparison differences according ANOVA test for bone cells in each durations

| Variables | Duration | Control group | Sponge group | RS group |
|-----------|----------|---------------|--------------|----------|
| Osteoblast | 2 weeks | 20.1 ± 1.35 | 30.6 ± 2.21 | 29.7 ± 2.5 |
|           | 4 weeks  | 30.8 ± 3.03 | 39.2 ± 3.49 | 36.8 ± 2.9 |
| Osteocytes| 2 weeks | 25.6 ± 2.38 | 33.5 ± 2.39 | 26.7 ± 2.3 |
|           | 4 weeks  | 35.6 ± 2.98 | 37.8 ± 2.49 | 29.8 ± 2.7 |
| Osteoclasts| 2 weeks | 0.44 ± 0.41 | 0.35 ± 0.55 | 0.38 ± 0.49 |
|          | 4 weeks  | 0.09 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 |

Table: Group (4) comparison differences according ANOVA test for bone cells in each durations

| Variables  | Duration | t-test | p-value |
|------------|----------|--------|---------|
| Osteoblasts| 2 weeks  | 18.35  | 0.0001**|
|            | 4 weeks  | 16.56  | 0.0001**|
| Osteoclasts| 2 weeks  | 3.2    | 0.003**|
|            | 4 weeks  | 1.44   | 0.03*   |
| Osteocytes | 2 weeks  | 27.21  | 0.0001**|
|            | 2 weeks  | 31.82  | 0.0001**|

* Significant difference (p < 0.05)
** High Significant difference (p < 0.01)
structure and course of action inside the interface with the extracellular grid or with an embedded material [15]. While in red clover oil and sponge group (GS) treated defect area showed more and thicker bone trabeculae than that of other groups. It has been stated that isoflavones compounds could increase the osteogenic effect by increasing the osteoblast cell proliferation and stimulating matrix activity. A study conducted by [16].

A previous study [17] has been performed to evaluate the performance of isoflavones compounds as a scaffold in bone regeneration procedures to be a promoter of osteoblastic formation and is readily resorbed by osteoclasts. Direct bone matrix anchorage has been shown with collagen fibers deposited in the micropores. resorption of isoflavones compounds has been reported in a rabbit model with cells having a characteristic of osteoclast cells activity. Histomorphometry permits quantitative examination of histological information, to be specific with respect to length and separation, territory and number of the segments of intrigue [18].

Mean values of trabecular area and number recorded in this study were higher in Combination (RS) group, more clearly observed in 4 weeks duration which may seem to be in line with histomorphometric results of Occhiuto et al. 2007 [19], who stated that the increase in osteogenesis seen during the transition from the 14 to 30 days of observation and the total areas of the newly created bone trabeculae showed that the groups submitted to bone filling biomaterial (osteocconduct and autogenous bone graft) showed bone trabeculate area values higher than the control group of the same animal.

The present study revealed that the numbers of osteoblasts was highest in combination (RS) group when compared to others especially in 2 weeks interval. While the number of osteocytes increase with time in all studied groups especially in combination group at 4 weeks interval. These results could be explained by the direct action of red clover oil on the differentiation and maturation of osteoblasts and accelerating rate of matrix deposition and its corresponding calcification. These finding agree with Bharathi, & Baby; 2017 [20] who found that isoflavones compounds could increase the rate of bone ossification, it is thought to affect bone metabolism by promoting the proliferation of osteoblasts and the synthesis of osteon, which leads to the inhibition of the differentiation of osteoclast-like cells.

Conclusions:
Red clover oil is osteoinductive herbal material that promote and accelerates bone healing process by an early bone formation and maturation. Histomorphometric parameters for all groups showed highly significant difference in overall indicators of bone micro architectures include trabecular area, trabecular number, bone marrow space area, osteoblasts, and osteocytes numbers.

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