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Original Article

Effect of Eucommia Ulmoides on Healing of Bon Defect Using Histological and Histomorphometric Analysis in Rat: *in vivo* Study

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Abstract

Bone repair is a complex multistep process. The flavonoid group present in Eucommia ulmoides (EU) helps to increase bone mineral density. This study aimed to evaluate the healing process of bone defects treated with EU and compare it to the control group using histological and histomorphometric tests. For this purpose, 24 albino rats were anesthetized and both of their femurs were prepared by drilling intra-bony defects (2 mm in diameter and 3 mm in depth). In each rat, the right bony defects were considered control, while the left bony defects were treated with EU. Moreover, scarification was done with 1-, 2-, and 4-week healing intervals (n=8). Histological and histomorphometric analysis of bone microarchitectures were performed for more evaluations and the bone cells were counted (osteoblast, osteocyte, and osteoclast) for comparison with the normal percentages. Moreover, trabecular number, trabecular area, and bone marrow area per mm^2 were measured using the ImageJ software. The recorded histological data revealed the acceleration of bone healing in the EU group, compared to the control group. Highly significant differences were observed in the animals treated with EU, compared to the control group for almost all histomorphometric parameters investigated in this Study. In conclusion, EU can improve bone healing and increase osteogenic capacity in rats.

Keywords: Bone healing, Eucommia Ulmoides, ImageJ

1. Introduction

Bone healing is a remarkably complex reformative process. Bone tissue commonly heals spontaneously unless there are complicated circumstances, such as large bone defects (1). Bone healing can be divided into three phases, namely inflammatory, reparative, and remodeling (2).

Eucommia ulmoides (EU) (commonly called "Du Zhong" in the Chinese language) belong to the family of Eucommiaceae, a genus of the small tree, native to Central China. This plant is widely cultivated in China on a large scale due to its medicinal importance. About 112 compounds have been isolated from the EU, which include lignans, iridoids, phenolics, steroids, and other compounds. The leaf of the EU has higher activity, compared to its cortex, flower, and fruit. The leaves of the EU have been reported to enhance bone strength and body muscles, thereby leading to longevity and the promotion of fertility in humans (3). About 29 phenolic compounds have been isolated and identified from the EU (4). Six steroids and five terpenoids have been extracted and categorized from the EU (5). As observed by malonaldehyde and glutathione levels after renal perfusions, polysaccharides from EU were reported to exhibit protective effects on kidneys (6). Amino acids, microelements, vitamins, and fatty acids have also been isolated from the EU (7).

Eucommia ulmoides can be used in the control of osteoporosis since Eucommia extract is actively involved in mechanisms that initiate osteoblast

formation, enhance osteogenesis, decrease osteoclast, and thereby prevent osteolysis (8). Antioxidant compounds of the Eucommia plant reduce the level of free radicals and improve the disease condition caused by oxidative stress (9). Moreover, it has been well documented in previous studies that Eucommia plant extract has antibacterial, antiviral, and anti-inflammatory activities; therefore, it can inhibit the growth of bacteria and reduce the secretion of proinflammatory cytokines (10).

The present study aimed to evaluate the healing process of bone defects treated with EU and compare it with the control group using histological and histomorphometric analyses.

2. Materials and Methods

2. 1. Animal Preparation

Supervision and nursing were provided by the staff of the animal house at Kufa University, Najaf, Iraq. This experimental study was performed on 24 male albino rats that were 6-8 months old and weighed 250-400 g. The animals were anesthetized, and surgical operations were performed on the distal side of the right and left femurs for induction of two intra-bony defects of about 3 mm in depth and 2 mm in width in each femur of all rats. The exposed femur bone can be seen in figures 1 and 2.

Figure 1. Making bone defect

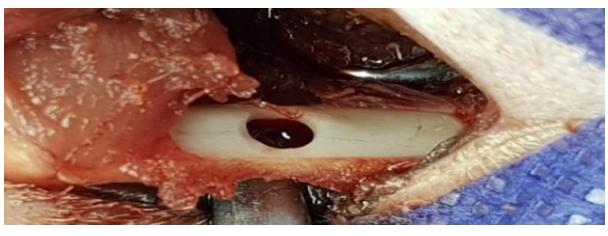

Figure 2. Bone defect

Scarification of animals was carried out by giving an overdose of anesthesia 1, 2, and 4 weeks postoperatively (8 rats for each healing interval). The bone specimen was prepared by cutting the bone about 5 mm away from the operation site and stored in 10% freshly prepared formalin.

2. 2. Histological Preparation

Bony specimens were fixed with 10% freshly prepared buffered formalin for 24 h. Afterward, the decalcification process started using 10% formic acid for 3-8 days and embedding was performed using paraffin wax. The bone blocks were sectioned by microtome for serial sections of 4 μm that were taken and placed on a slide. Staining was performed using hematoxylin and eosin stains. Histomorphometric analysis of bone microarchitectures was performed and the number of bone cells (osteoblast, osteocyte, and osteoclast) per mm^2 was counted. In addition, trabecular number, trabecular area, and bone marrow area per mm^2 were measured using the ImageJ software.

2. 3. Statistical Analysis

The collected data were analyzed using SPSS software (version 26). Independent t-test and Analysis of Variance (ANOVA) were used to compare the continuous variables accordingly. A *P* value of less than 0.05 was considered statistically significant.

3. Results

Almost all histological sections showed a suitable healing pathway for all experimental and control groups, but with a difference in rate regarding their healing intervals. In one week after the operation, the control group showed granulation tissue and some osteoblast (Figure 3), whereas the EU group showed bone spicules coalesce with some cutting bone (Figure 4). In two weeks, control and experimental groups revealed new bone trabecula rimmed by active osteoblasts, filled with osteocytes, and demarcated from basal bone by a reversal line (Figures 5 and 6). Four weeks after the operation, the defect site in control groups showed thick bone trabecula with regression of

bone marrow space and the presence of osteoclast and osteoblast (Figure 7). However, in the experimental group, the bony defect was filled with mature bone by the presence of haversian lamella (osteon) (Figure 8).

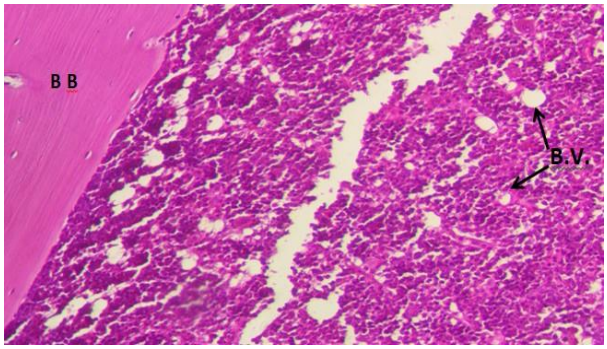

Figure 3. Defect area at one week control group shows newly osteoid tissue and osteoblast, and inflammatory cells (I C) with newly formed blood vessels (BV) surrounded by basal bone (BB) H&E ×20

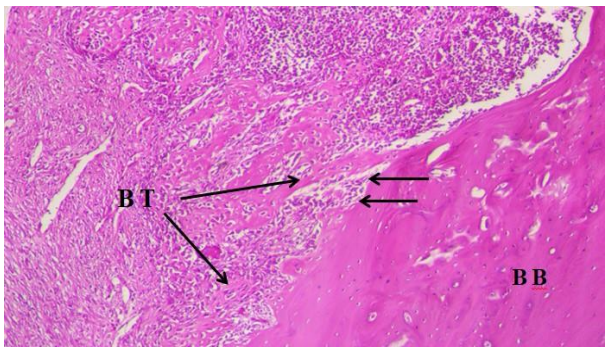

Figure 4. View of one-week defect area treated with Eucommia ulmoides shows new thin bone trabeculae (BT) separated from basal bone (BB) by reversal line (arrows). H&E ×10

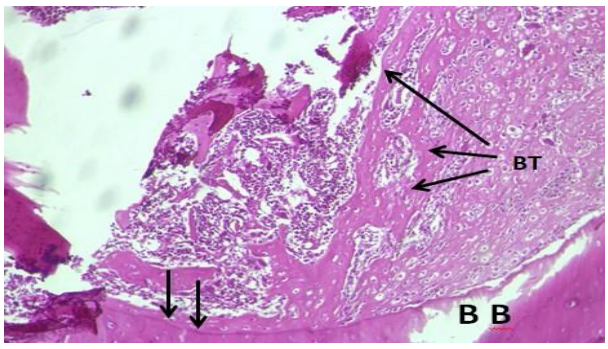

Figure 5. Microphotograph view of 2 weeks control defect area shows new bone trabeculae (BT), which are demarcated from basal bone (BB) by reversal line (arrows) H&E×10

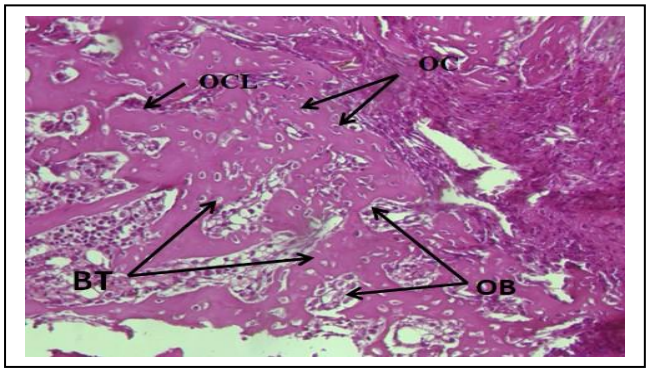

Figure 6. View of the 2-week experimental group shows new bone trabeculae (BT) surrounded by osteoblast (OB) and osteoclast (OCL), and osteocyte (OC) H&E ×20

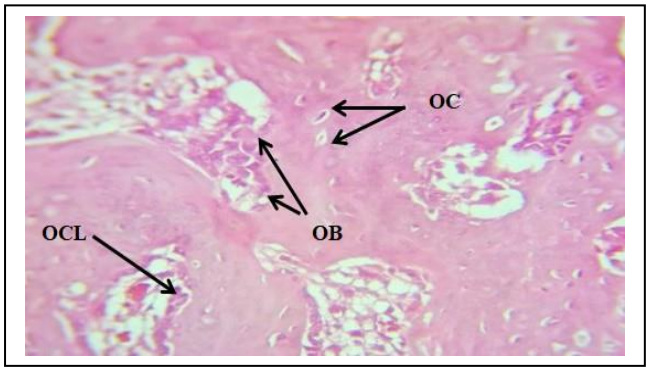

Figure 7. View of four weeks control group shows bone trabeculae (BT) with osteoblast (OB), osteocyte (OC), and osteoclast (OCL) H&E ×40

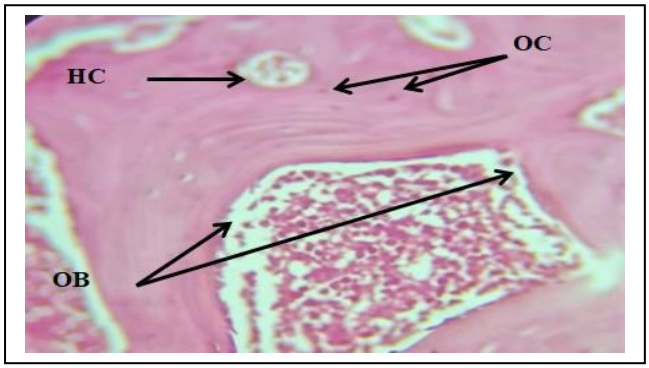

Figure 8. View of four weeks experimental group shows osteoblast (OB), osteocytes (OC) show regular surround. Haversian canal (HC) H&E ×20

Tables 1-4 summarize the results of the t-test comparison in terms of the mean of osteoblast, osteocyte, trabecular area, and bone marrow area, respectively, with 1-, 2-, and 4-week intervals in both groups. The results showed a higher mean value for osteoblast in the experimental group after 2 weeks, for osteocyte and trabecular area in the experimental group after 4 weeks, and for bone marrow in the control group after 2 weeks.

Tables 5 and 6 tabulated the results of the comparison of different durations by ANOVA test in terms of the mean number of osteoblasts and osteocytes, respectively. In these tables the most highly significant difference among 1-, 2-, and 4-week intervals in terms of the osteoblast and osteocyte in both groups are presented. Moreover, Tables 7 and 8 summarize the results of the t-test in terms of the different durations for the trabecular and bone marrow areas, respectively. Both groups showed a highly significant duration difference between 2- and 4-week intervals.

Table 1. T-test group Comparison difference in mean of osteoblasts at 1, 2, and 4 weeks duration

Week	N	Osteoblast		Range	T-test	*P*-value
		Experiment Mean±SD	Control Mean±SD			
1 Week	8	14.0±2.26	10.12±2.0	8.0-17.0	3.657	0.003
2 Week	8	18.62±1.6	15.5±2.4	12.0-22.0	3.022	0.011
4 Week	8	15.37±1.2	12.87±1.8	11.0-17.0	3.269	0.007

Table 2. T-test group Comparison difference in mean of osteocytes at 1, 2, and 4 week's duration

Week	N	Osteoblast		Range	T-test	*P*-value
		Experiment Mean±SD	Control Mean±SD			
1 Week	8	7.87±1.1	4.5±1.6	2.0-9.0	4.872	0.001
2 Week	8	12.25±1.0	7.75±1.0	6.0-14.0	8.695	0.001
4 Week	8	17.0±1.7	14.37±2.1	12.0-20.0	2.727	0.017

Table 3. T-test group Comparison difference in mean of the trabecular area at 2 and 4 weeks duration

Week	N	Osteoblast		Range	T-test	*P*-value
		Experiment Mean±SD	Control Mean±SD			
2 Week	8	0.152±0.01	0.111±0.003	0.106-0.161	16.853	0.001
4 Week	8	0.176±0.01	0.12±0.003	0.116-0.191	16.229	0.001

Table 4. T-test group Comparison difference in mean of bone marrow area at 2 and 4 weeks duration

Week	N	Osteoblast		Range	T-test	*P*-value
		Experiment Mean±SD	Control Mean±SD			
2 Week	8	0.064±0.01	0.137±0.02	0.056-0.0156	11.441	0.001
4 Week	8	0.041±0.01	0.119±0.01	0.031-0.128	27.367	0.001

Table 5. Duration comparison difference by ANOVA-test in osteoblasts in each group

Wound	Osteoblast			F-value	*P*-value
	Week 1 Mean±SD	Week 2 Mean±SD	Week 4 Mean±SD		
Experiment	14.0±2.3	18.62±1.6	15.37±1.2	14.865	0.001
Control	10.12±2.0	15.5±2.4	12.87±1.8	13.228	0.001

Table 6. Duration comparison difference by ANOVA- test in osteocytes in each group

Wound	Osteoblast			F-value	*P*-value
	Week 1 Mean±SD	Week 2 Mean±SD	Week 4 Mean±SD		
Experiment	7.87±1.1	12.25±1.0	17.0±1.7	96.196	0.001
Control	4.5±1.6	7.75±1.0	14.37±2.1	74.163	0.001

Table 7. Duration comparison difference by T-test in the trabecular area in each group

Trabecular area	Follow Up		*P*-value
	Two weeks Mean±SD	Four weeks Mean±SD	
Experiment	0.152±0.01	0.176±0.01	0.001
Control	0.111±0.003	0.12±0.003	0.001

Table 8. Duration comparison difference by T-test in bone marrow area in each group

Trabecular area	Follow Up		*P*-value
	Two weeks Mean±SD	Four weeks Mean±SD	
Experiment	0.064±0.01	0.041±0.01	0.001
Control	0.137±0.02	0.119±0.01	0.001

4. Discussion

At the microscopic level, bone remodeling is performed in basic multicellular units, where osteoclasts resorb a certain quantity of bone and osteoblasts from the osteoid matrix and mineralize it to fill the previously created cavity. Postoperative histological findings revealed the deposition of new bone in the defect site in almost all experimental and control groups, but at different rates in each group. Bone healing progression indicated by bone matrix deposition and trabecular bone formation increased in thickness with time. Osteoclasts resided in Howship's lacunae at trabecular bone borders, indicating a remodeling process, especially 2 weeks after the operation in both groups. Histomorphometric analysis revealed that bone marrow space area decreased with time, which was evident in all groups.

The histological examination of bone sections showed deposition of osteoid bone by osteoblast in all studied groups after 1 week. Moreover, small bone spicules rimmed by osteoblasts and filled with large-size osteocytes were observed more clearly in experimental groups. The results of this study are consistent with those of a study conducted by Liang, Yu (11), who found that EU promoted osteoblast proliferation and increased alkaline phosphatase, collagen I, osteocalcin,

and TGFβ1 levels of both mRNA and protein in osteoblasts.

Microscopic examination of serial sections from two weeks after the operation in the intervention site showed that the new bone trabecular formation in both groups and the experimental group highly increased the number of osteoblast and osteocytes, compared to the control group. This result was in line with those of a study carried out by Liang, Yu (11) who examined bone sections of the control group after 2 weeks. Zhang, Ravipati (10) stated that EU could increase the osteogenic effect by increasing osteoblast cell proliferation and stimulating matrix activity.

Four weeks after the operation, the results of the present study revealed that dense bone trabecular filled the bone defect site in the control group. However, in the experimental group treated by EU, mature bone was seen by the presence of haversian lamellae which concentrated around the haversian canal by the arrangement of osteocytes. This finding is inconsistent with those of a study performed by Hendra, Ahmad (9) which showed that after 4 weeks, more areas of bone formation were observed with thin and small bony trabeculae.

Results of the present study illustrated that the mean values of the studied parameters, including trabecular area trabecular number, underwent an increase in the experimental group, compared to the control group after the 4-week interval. Moreover, it was found that the mean value of bone marrow decreased due to bone maturation increasing with time. In addition, there was a highly significant difference between both groups after 2- and 4-week healing intervals. Effects of local EU administration on bone healing might induce more trabecular bone and higher trabecular area density.

Treatment of the bony defect by EU improves bone healing and increases osteogenesis capacity. This could be explained by the direct effect of EU on the differentiation and maturation of osteoblasts and then the acceleration of the rate of matrix deposition and its corresponding calcification.

Authors' Contribution

Study concept and design: H. O. J.
Acquisition of data: N. M. H. A.
Analysis and interpretation of data: H. O. J.
Drafting of the manuscript: H. O. J.
Critical revision of the manuscript for important intellectual content: N. M. H. A.
Statistical analysis: N. M. H. A.
Administrative, technical, and material support: H. O. J.

Ethics

All experimental procedures were conducted in accordance with the ethical approval of animal experiments of the College of Dentistry, University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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