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Original Article

Histological evaluation of effects of krill oil and eucalyptus oil on bone healing in rats

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الملخص

أهداف البحث: لا يزال إصلاح عيوب العظام يمثل تحديا كبيرا في طب العظام. يهدف هذا البحث إلى تقييم فعالية تطبيق زيت الكريل موضعيا كمحفز عظمي في التئام العظام. ستفحص الدراسة تأثير زيت الكافور كمنشط لشفاء عيوب العظام.

طريقة البحث: استخدمت هذه الدراسة ثمانية وأربعين من ذكور الجرذان البيضاء التي تزن بين ٣٠٠ و٤٠٠ غرام وتتراوح أعمارها بين ستة وثمانية أشهر. تمت مراقبة بيئتها، بما في ذلك كمية الماء والطعام الذي تتناوله، بعناية. سيكون الجانب الأيمن من ظنبوب الجرذ هو موقع التجربة، بينما سيكون الجانب الأيسر هو جانب التحكم. ستُجرى العملية على الجوانب الداخلية من ظنبوب الجرذ. سيتم معالجة عيب العظام في مجموعة التحكم بتطبيق ١ ميكرومتر من الماء المقطر موضعيا مرة واحدة يوميا. في المجموعة التجريبية، ستكون هناك ثلاث مجموعات فرعية: المجموعة الأولى ستتلقى تطبيقا موضعيا يوميا من ١ ميكرومتر من زيت الكريل، المجموعة الثانية ستتلقى تطبيقا موضعيا يوميا من ١ ميكرومتر من زيت الكافور، والمجموعة الثالثة ستتلقى تطبيقا موضعيا يوميا من ١ ميكرومتر من مزيج زيت الكريل وزيت الكافور. سيتم التضحية بالجرذان بعد ٧ و١٤ يوما من الجراحة، مع ستة جرذان لكل فترة واثني عشر جرذا لكل مجموعة.

النتائج: لوحظ تطور الأنسجة الشبيهة بالعظام وزيادة كبيرة في عدد خلايا العظام بعد الإعطاء الموضعي لزيت الكريل أو زيت الكافور وحدهما، أو كلا المادتين معا، على إصابة الظنبوب. كان هناك تغير ملحوظ في منطقة نخاع العظم، وزيادة في مساحة العظام الحويجزية وانخفاض تدريجي في عدد العظام الحويجزية.

الاستنتاجات: في الختام، هناك وعد علاجي في الجمع بين زيت الكريل وزيت الكافور لتسريع إصلاح تشوهات العظام. تتحكم زيوت الكريل والكافور في عمليات تكوين العظام وتكوين الأوعية الدموية.

الكلمات المفتاحية: تكوين الأوعية الدموية؛ زيت الكريل؛ مشكل العظام؛ زيت الكافور؛ تكوين العظام.

Abstract

Background: Bone defect repair is a significant challenge in orthopedic medicine.

Objectives: This study assessed the effectiveness of applying krill oil locally as an osteoinducer in bone healing, and the impact of eucalyptus oil as a stimulant for healing bone defects.

Subjects and methods: In this study, 48 albino male rats weighing 300–400 g and aged 6–8 months were used, where their environment was carefully monitored, including the amounts of water and food consumed. The experimental test site was the side of the right tibia and the control site was on the left tibia. The treatment was performed on the inner side of the tibia. Bone deficit treatment on the control site involved applying 1 μL of distilled water topically once each day. The experimental treatments were tested in three subgroups: group I received daily local application with 1 μL of krill oil, group II received daily local application with 1 μL of eucalyptus oil, and group III received daily local application with 1 μL of-a combination of krill oil and eucalyptus oil. Six rats were sacrificed at 7 and 14 days after surgery, with 12 rats in each group.

Results: Osteoid tissue development and dramatic increases in the numbers of bone cells were observed after local administration of either krill oil or eucalyptus oil

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separately, or both substances combined, to a tibia lesion. Notable changes in the area of bone marrow occurred, with increases in the trabecular bone area and gradual decreases in the trabecular bone number.

Conclusion: Combined treatment with krill oil and eucalyptus oil has therapeutic potential for accelerating the mending of bone deformities. Krill and eucalyptus oils can affect osteogenesis and angiogenesis processes.

Keywords: Angiogenesis; Bone morphogenetic; Eucalyptus oil; Krill oil; Osteogenesis

Introduction

Bones are strong structures that constitute most of the internal skeleton in vertebrates. Organs rely on bones for support, and they also have roles on defense, mineral storage, and the erythrocyte and lymphocyte maturation processes.[1] (see Tables 1−19).

The bone healing process occurs in three primary stages. First, in the inflammation stage, a hematoma develops as a result of tissue damage at the wounded site. Bony necrosis then develops around the margins of the fracture due to thrombosis in nearby blood vessels. Mesenchymal stem cells are then promoted to proliferate and differentiate by osteoinductive growth factors due to the formation of a local inflammatory milieu caused by increased capillary permeability. This stage typically lasts from one to seven days.[2]

In the repair stage, a periosteal callus develops around the edges of the damage (preosteoblasts initiate intramembranous ossification). Endochondral ossification occurs at the location inside the fracture's hematoma and an intramedullary callus develops at the fracture's midpoint. Chemical and mechanical factors promote the formation and mineralization of the callus.[3]

In the remodeling stage, the woven bone transitions into lamellar bone over time, and the medullary cavity is reformed.[3]

Krill oil is a dietary supplement made from Antarctic krill, *Euphausia superba*. This supplement contains phospholipid-derived fatty acids in the same manner as omega-3 fatty acids in fish oil. US Food and Drug officials have certified krill oil as "generally recognized as safe" (GRAS), although fish and krill from Antarctica may contain harmful residues. Krill oil is also included in the European Union's approved list of innovative foods.[4]

Eucalyptus oil sourced from the native Australian eucalyptus tree and grown globally, is a versatile substance with a variety of uses, including pharmaceutical, antiseptic, repellent, flavoring, fragrance, and industrial applications. The oil is obtained through steam distillation from selected *Eucalyptus* species.[5]

Table 1: Descriptive statistics of inflammatory cell count between groups at 7 day duration.

Descriptive Statistics						
	Tested groups	N	Minimum	Maximum	Mean	Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	17.5	20	18.625	0.55	1.11
Krill oil	4	13	19	15.25	1.31	2.62
Eucalyptus	4	16	20	18	0.81	1.63
Combination	4	11	18	14	1.47	2.94

P value 0.001 sig.

Table 2: Paired samples T-Test between the groups at 7 day duration regarding inflammatory cell count.

Paired Samples T-Test									
Tested groups		Paired differences					t	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	3.38	1.70	0.85	0.67	6.08	3.97	3	0.029
Pair 2	Control group − eucalyptus	0.63	0.75	0.38	−0.57	1.82	1.67	3	0.194
Pair 3	Control group − combination	4.63	2.29	1.14	0.99	8.26	4.05	3	0.027
Pair 4	Krill oil - eucalyptus	−2.75	1.71	0.85	−5.47	−0.03	3.22	3	0.049
Pair 5	Krill oil - a combination	1.25	0.96	0.48	−0.27	2.77	2.61	3	0.080
Pair 6	Eucalyptus - combination	4.00	2.45	1.22	0.10	7.90	3.27	3	0.047

$P \leq 0.05$ is considered statically significant.

Table 3: T-Test of osteoblast cell count between the groups at 7 day duration.

Paired Samples T-Test/bone cell count 7 days									
Tested groups osteoblast		Paired dDifferences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−9.25	3.10	1.55	−14.18	−4.32	5.98	3	0.009
Pair 2	Control group - eucalyptus oil	−2.75	2.63	1.31	−6.93	1.43	2.09	3	0.128
Pair 3	Control group - combination	−14.75	2.50	1.25	−18.73	−10.77	11.80	3	0.001
Pair 4	Krill oil - eucalyptus oil	6.50	0.58	0.29	5.58	7.42	22.52	3	0.000
Pair 5	Krill oil - a combination	−5.50	1.29	0.65	−7.55	−3.45	8.52	3	0.003
Pair 6	Eucalyptus oil - combination	−12.00	1.15	0.58	−13.84	−10.16	20.79	3	0.000

$P \leq 0.05$ is considered statically significant.

Table 4: Descriptive statistic of osteoblast cell count at 14 day duration Descriptive Statistics/bone cell count 14 days.

Tested groups osteoblast	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	35	41	38.5	1.32	2.65
Krill oil	4	46	50	48	0.91	1.83
Eucalyptus oil	4	43	47	45	0.91	1.83
Combination	4	49	50	49.5	0.28	0.57

P value 0.001 sig.

Table 5: Paired samples T-Test osteoblast cell count at 14 day duration.

Paired Samples T-Test/bone cell count 14 days									
Tested groups osteoblast		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−9.50	1.00	0.50	−11.09	−7.91	19.00	3	0.000
Pair 2	Control group eucalyptus oil	−6.50	1.73	0.87	−9.26	−3.74	7.51	3	0.005
Pair 3	Control group -combination	−11.00	2.16	1.08	−14.44	−7.56	10.18	3	0.002
Pair 4	Krill oil - eucalyptus oil	3.00	0.82	0.41	1.70	4.30	7.35	3	0.005
Pair 5	Krill oil - a combination	−1.50	1.29	0.65	−3.55	0.55	2.32	3	0.103
Pair 6	Eucalyptus oil - combination	−4.50	1.29	0.65	−6.55	−2.45	6.97	3	0.006

$P \leq 0.05$ is considered statically significant.

Table 6: Comparisim between duration regarding osteoblast cell count Osteoblast 7 days vs 14 days.

Time duration/Tested groups	control group	krill oil	eucalyptus oil	combination
Mean ± SD/7 days	C	B	C	A
	32.3 ± 2.1	41.5 ± 1.3	35 ± 0.8	47 ± 0.8
Mean ± SD/14 days	C	A	B	A
	38.5 ± 2.6	48 ± 1.8	45 ± 0.8	49.5 ± 0.9
P value	0.002	0.001	0.001	0.09

LSD test was used to calculate the significant differences between the tested mean, the letters (A, B, and C) represented the levels of significant, and highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean. Values of $p \leq 0.05$ were considered significantly different.

Table 7: Descriptive statistics of osteocyte cell count at 7 day duration.

Descriptive Statistics/bone cell count 7 days					
Tested groups osteocyte	N	Minimum	Maximum	Mean	Std. Deviation
	Statistic	Statistic	Statistic	Statistic Std. Error	Statistic
Control group	4.00	35	38	36.5 0.65	1.29
Krill oil	4.00	43	46	44.5 0.65	1.29
Eucalyptus oil	4.00	36.	39	37.25 0.75	1.5
Combination	4.00	47	50	48.50 0.65	1.29

P value 0.001 sig.

Table 8: Paired samples T-Test for osteocyte cell count at 7 day duration.

Paired Samples T-Test/bone cell count 7 days									
Tested groups Osteocyte		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−8.00	0.82	0.41	−9.30	−6.70	19.60	3	0.001
Pair 2	Control group - eucalyptus oil	−0.75	0.50	0.25	−1.55	0.05	3.00	3	0.06
Pair 3	Control group - combination	−12.00	1.41	0.71	−14.25	−9.75	16.97	3	0.001
Pair 4	Krill oil - eucalyptus oil	7.25	0.50	0.25	6.45	8.05	29.00	3	0.001
Pair 5	Krill oil - a combination	−4.00	1.63	0.82	−6.60	−1.40	4.90	3	0.02
Pair 6	Eucalyptus oil - combination	−11.25	1.71	0.85	−13.97	−8.53	13.17	3	0.001

$P \leq 0.05$ is considered statically significant.

Table 9: Descriptive Statistics of osteocyte cell count at 14 days.

Descriptive Statistics/bone cell count 14 days					
Tested groups osteocyte	N	Minimum	Maximum	Mean	Std. Deviation
	Statistic	Statistic	Statistic	Statistic Std. Error	Statistic
Control group	4	40	44	42.25 0.85	1.71
Krill oil	4	50	52	51.00 0.41	0.82
Eucalyptus oil	4	46	49	47.75 0.75	1.50
Combination	4	50	55	53.00 1.08	2.16

P value 0.001 sig.

Table 10: Paired samples T-Test of osteocyte cell count at 14 day duration.

Paired Samples T-Test/bone cell count 14 days									
Tested groups Osteocyte		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−8.75	1.50	0.75	−11.14	−6.36	11.67	3	0.001
Pair 2	Control group - eucalyptus oil	−5.50	2.38	1.19	−9.29	−1.71	4.62	3	0.02
Pair 3	Control group - combination	−10.75	0.96	0.48	−12.27	−9.23	22.46	3	0.001
Pair 4	Krill oil - eucalyptus oil	3.25	1.26	0.63	1.25	5.25	5.17	3	0.01
Pair 5	Krill oil - a combination	−2.00	1.83	0.91	−4.91	0.91	2.19	3	0.12
Pair 6	Eucalyptus oil - combination	−5.25	2.22	1.11	−8.78	−1.72	4.74	3	0.02

$P \leq 0.05$ is considered statically significant.

Table 11: comparison of durations regarding osteocyte cell count Osteocytes 7 days vs 14 days.

Time duration/Tested groups	control group	krill oil	eucalyptus oil	combination
Mean ± SD/7 days	C 36.5 ± 1.3	B 44.5 ± 1.3	C 37.25 ± 1.5	A 48.5 ± 1.3
Mean ± SD/14 days	C 42.25 ± 1.7	A 51 ± 0.8	B 47.75 ± 1.5	A 53 ± 1.2
P value	0.05	0.05	0.003	0.05

LSD test was used to calculate the significant differences between the tested mean, the letters (A, B, and C) represented the levels of significant, and highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean. Values of $p \leq 0.05$ were considered significantly different.

Table 12: Descriptive statistics of osteoclast cell count at 7 day duration.

Descriptive Statistics/bone cell count 7 days						
Tested groups osteoclast	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	0.0	1.0	0.5	0.3	0.6
Krill oil	4	1	2.0	1.3	0.3	0.5
Eucalyptus oil	4	0.0	1.0	0.8	0.3	0.5
Combination	4	1	2.0	1.5	0.3	0.6

P value 0.001 sig.

Table 13: Paired samples T-Test of osteoclast cell count at 7 day duration.

Paired Samples T-Test/bone cell count 7 days									
Tested groups Osteoclast		Paired differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−0.75	0.96	0.48	−2.27	0.77	1.57	3	0.215
Pair 2	Control group - eucalyptus oil	−0.25	0.50	0.25	−1.05	0.55	1.00	3	0.391
Pair 3	Control group - combination	−1.00	0.82	0.41	−2.30	0.30	2.45	3	0.092
Pair 4	Krill oil - eucalyptus oil	0.50	1.00	0.50	−1.09	2.09	1.00	3	0.391
Pair 5	Krill oil - a combination	−0.25	0.96	0.48	−1.77	1.27	0.52	3	0.638
Pair 6	Eucalyptus oil - combination	−0.75	0.50	0.25	−1.55	0.05	3.00	3	0.05

$P \leq 0.05$ is considered statically significant.

Table 14: Descriptive Statistics of osteoclast cell count at 14 days.

Descriptive Statistics/bone cell count 14 days						
Tested groups osteoclast	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	1	2	1.5	0.29	0.58
Krill oil	4	1	2	1.5	0.29	0.58
Eucalyptus oil	4	1	2	1.75	0.25	0.50
Combination	4	2	3	2.75	0.25	0.50

P value 0.002 sig.

Materials and Methods

The treatments used in this study were krill oil (supplied by amazon.com) and eucalyptus oil (supplied by amazon.com).

Surgical procedure

The experimental procedures followed ethical principles of animal experimentation number 802 on 19-2-2023. Treatments were tested on 48 albino male rats weighing 300–

Table 15: Comparisim of duration regarding osteoclast cell count.

Osteoclast 7 days vs 14 days				
Time duration/Tested groups	control group	krill oil	eucalyptus oil	combination
Mean ± SD/7 days	C 0.5 ± 0.58	A 1.25 ± 0.5	C 0.75 ± 0.5	A 1.5 ± 0.58
Mean ± SD/14 days	C 1.5 ± 0.58	C 1.5 ± 0.58	B 1.75 ± 0.5	A 2.75 ± 0.5
P value	0.05	0.87	0.03	0.04

LSD test was used to calculate the significant differences between the tested mean, the letters (A, B, and C) represented the levels of significant, and highly significant start from the letter (A) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean. Values of $p \leq 0.05$ were considered significantly different.

Table 16: Descriptive statistics of trabecular number at 14 day duration.

Descriptive Statistics						
Tested groups trabecular number	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	4	5	10	7.25	1.11
Krill oil	4	4	10	13	11.50	0.65
Eucalyptus oil	4	4	6	9	8.00	0.71
Combination	4	4	11	15	13.50	0.96

P value 0.01 sig.

Table 17: Paired samples of trabecular area at 14 day duration.

Paired Samples T-Test/Trabecular area 14 days									
Tested groups Trabecular area		Paired differences					T	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % confidence interval of the difference				
					Lower	Upper			
Pair 1	Control group - krill oil	−2.75	0.96	0.48	−4.27	−1.23	5.745	3	0.01
Pair 2	Control group - eucalyptus oil	−1.00	1.41	0.71	−3.25	1.25	1.414	3	0.252
Pair 3	Control group - combination	−5.25	1.50	0.75	−7.64	−2.86	7.000	3	0.006
Pair 4	Krill oil - eucalyptus oil	1.75	2.06	1.03	−1.53	5.03	1.698	3	0.188
Pair 5	Krill oil - a combination	−2.50	1.73	0.87	−5.26	0.26	2.887	3	0.063
Pair 6	Eucalyptus oil - combination	−4.25	1.50	0.75	−6.64	−1.86	5.667	3	0.011

$P \leq 0.05$ is considered statically significant.

Table 18: Descriptive Statistics of bone marrow area at 14 day duration.

Tested groups bone marrow area	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Control group	4	3	5	3.78	0.34	0.68
Krill oil	4	2	3	2.38	0.31	0.63
Eucalyptus oil	4	2	4	2.75	0.32	0.65
Combination	4	1	2	1.50	0.18	0.36

P value 0.003 sig.

Table 19: paired Samples T-Test of Bone marrow area at 14 days.

Tested groups Bone marrow area		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95 % Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Control group - krill oil	1.40	1.05	0.53	−0.27	3.07	2.662	3	0.076
Pair 2	Control group - eucalyptus oil	1.03	0.61	0.30	0.06	1.99	3.374	3	0.043
Pair 3	Control group - combination	2.28	0.94	0.47	0.78	3.77	4.843	3	0.017
Pair 4	Krill oil - eucalyptus oil	−0.38	1.25	0.63	−2.36	1.61	0.600	3	0.591
Pair 5	Krill oil - a combination	0.88	0.47	0.24	0.12	1.63	3.710	3	0.034
Pair 6	Eucalyptus oil - combination	1.25	0.97	0.49	−0.30	2.80	2.574	3	0.082

$P \leq 0.05$ is considered statically significant.

400 g and aged 6–8 months, which were housed in a controlled temperature environment, and the amounts of food and drink consumed were carefully monitored. Surgical operations were performed on the interior of the tibia bones, where the experimental treatment was applied on the right tibia bone and the control treatment on the left tibia bone. Distilled water was applied locally to the bone defect as the control treatment. The experimental treatments were tested in three subgroups: group I received daily local application with 1 µL of krill oil, group II received daily local application with 1 µL of eucalyptus oil, and group III received daily local application with 1 µL of a combination of krill oil and eucalyptus oil. In total, 12 rats were tested for each experimental treatment, where six animals were each euthanized at 7 and 14 days post-surgery.

Histological examination

Tissue samples were fixed in 10 % formalin for 24 h, before soaking in a mixture of sodium citrate and formic acid to dissolve the calcium. Every three to four days, new decalcification solution was applied to the specimens and they were needle-checked on a regular basis. Decalcification was complete when the specimen could be readily pierced with a needle. The specimens were then rinsed in water to remove any remaining acid before standard tissue processing. Histological examination was conducted using Image J software to assess the bone area and other metrics based on pictures acquired with a light microscope at 40×magnification. A highly skilled pathologist who was unaware of the sample groupings conducted the analysis to prevent bias.

Statistical analysis

Statistical analyses were performed using SPSS version 21 to analyze and assess the data based on the minimum, maximum, mean, and standard deviation values for quantitative variables. Statistical tables and graphical visualizations were prepared. The Student's *t*-test was applied to determine significant differences between groups. Paired-sample *t*-tests were conducted to detect differences in individuals and other paired measures in the groups examined. Differences in means for groups according to analysis of variance were confirmed by conducting post-hoc least significant difference tests. Significant differences were accepted at $P < 0.05$.

Results

Histological findings after 7 days

Control group

After 7 days, histological analyses for the control group showed that osteoid tissue, bony spicules, inflammatory cells, fibrous tissue, freshly created osteoblasts, and osteocytes all contributed to the development of new bone in the bone defect area (Figure 1).

Krill oil treatment group

Histological images obtained after treatment with krill oil for 7 days demonstrated the production of new bone at the defect site, as well as the presence of inflammatory cells,

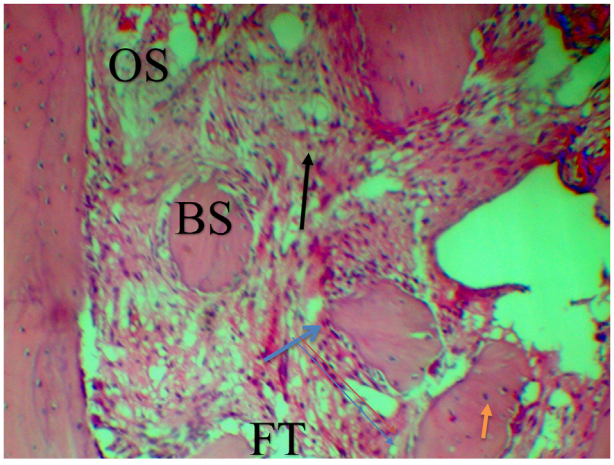

Figure 1: Histological view of the control group after 7 days showing the bone defect area. Osteoid tissue (OS), bony spicules (BS), inflammatory cells (black arrow), fibrous tissue (FT), newly formed osteoblasts (blue arrow), and osteocytes (orange arrow) contributing to new bone formation. H&E staining and 40× magnification.

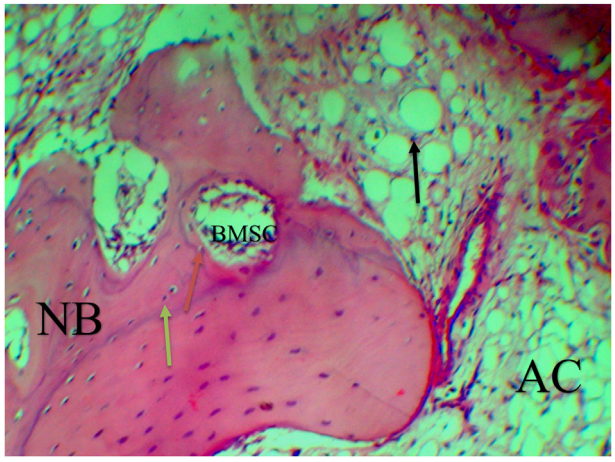

Figure 2: Histological image of the formation of new bone (NB) in the defect area after treatment with krill oil for 7 days showing the presence of osteoblasts (orange arrow), osteocytes (green arrow), inflammatory cells (black arrow), adipose cells (AC), and bone marrow stromal cells (BMSCs) surrounding the newly formed bone. H&E staining and 40× magnification.

adipose cells, osteoblasts, osteocytes, and bone marrow stromal cells surrounding the newly created bone (Figure 2).

Eucalyptus oil treatment group

Following treatment for 7 days with eucalyptus oil, histological analysis showed that bone trabeculae formed at the affected bone defect site. The bone matrix surrounding the injured site contained inflammatory, adipocyte, and new odontoblast cells (Figure 3).

Combined treatment group

After the combined treatment for 7 days, histological images indicated that osteoid tissue and new bone trabeculae were present in the defect area. These trabeculae were encased by newly formed bone stromal cells, and new

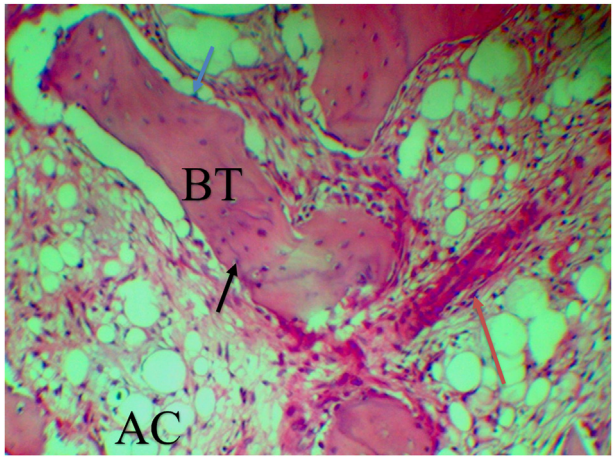

Figure 3: Histological image of the formation of bone trabeculae (BT) at the affected site after treatment with eucalyptus oil for 7 days showing the surrounding bone matrix containing adipocyte cells (AC), inflammatory cells (orange arrow), new odontoblasts (blue arrow), and osteocytes (black arrow). H&E staining and 40× magnification.

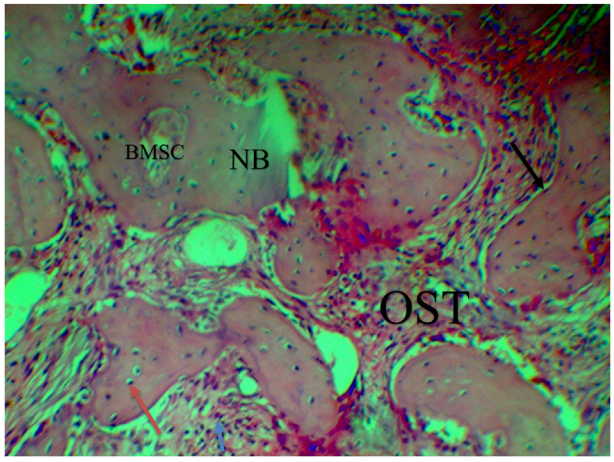

Figure 4: Histological image of ostoid tissue showing new bone trabeculae (NB) in the defect area after the combined treatment for 7 days. The trabeculae are surrounded by newly formed bone stromal cells, as well as new osteoblasts (black arrow) and osteocytes (orange arrow) embedded within the new bone trabeculae. Numerous inflammatory cells (blue arrow) are also visible in the newly formed bone trabeculae. H&E staining and 40× magnification.

osteoblasts and osteocytes were integrated within them. Furthermore, significant amounts of inflammatory cells were present within the newly formed bone trabeculae (Figure 4).

Histological findings after 14 days

Control treatment group

After 14 days under the control treatment, histological examination indicated the presence of recently developed bone, with bone marrow stromal cells encircled by osteoblasts and osteocytes incorporated into the newly formed bone (Figure 5).

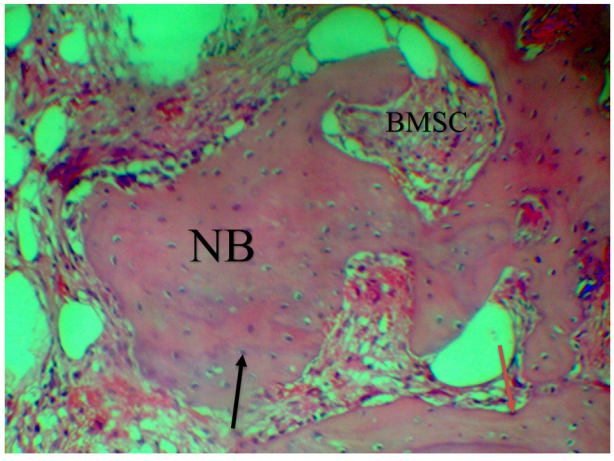

Figure 5: Histological image showing the presence of newly formed bone (NB), with bone marrow stromal cells (BMSC) surrounded by osteoblasts (orange arrow) and osteocytes (black arrow) embedded within the newly formed bone under the control treatment for 14 days. H&E staining and 40× magnification.

Krill oil treatment group

Following treatment with krill oil for 14 days, histological analysis demonstrated the development of fresh bone tissue with a new Haversian system, osteoblasts, osteocytes, newly formed blood vessels, and reversal lines suggesting the daily growth of new bone (Figure 6).

Eucalyptus oil treatment group

After treatment for 14 days with eucalyptus oil, histological examination indicated the presence of newly formed bone, newly developed osteoblasts, osteocytes, and osteoclasts. Furthermore, the observation of multiple reversal lines suggested the daily addition of newly formed bone (Figure 7).

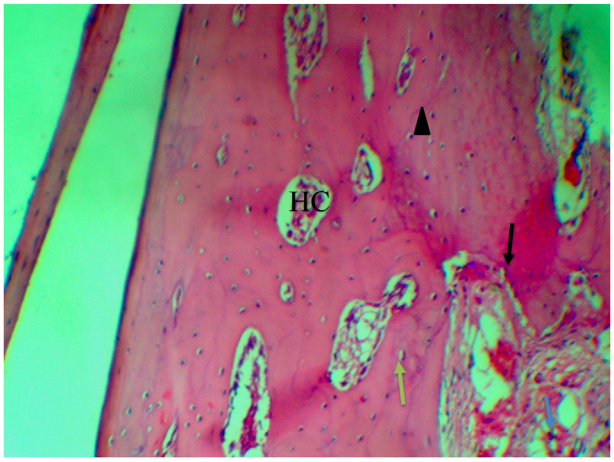

Figure 6: Histological image showing the presence of newly formed bone with a new Haversian system (HC), osteoblasts (black arrow), osteocytes (green arrow), newly formed blood vessels (blue arrow), and reversal lines (arrow head) indicating daily apposition of newly formed bone under treatment with krill oil for 14 days. H&E staining and 40× magnification.

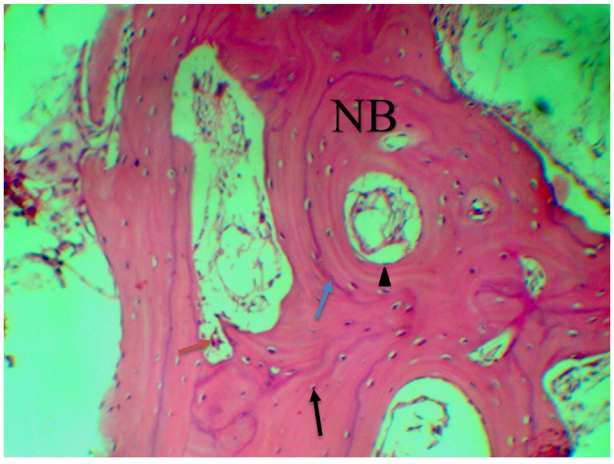

Figure 7: Histological image showing the presence of newly formed bone (NB), newly developed osteoblasts (arrow head), osteocytes (black arrow), and osteoclasts (orange arrow) treatment with eucalyptus oil for 14 days. The numerous reversal lines (blue arrow) indicate the daily apposition of newly formed bone. H&E staining and 40× magnification.

Combined treatment group

After the combined treatment for 14 days, histological examination demonstrated the presence of fully developed bone, recently formed osteoblasts, osteocytes, developing blood vessels, and osteons, which all suggested mature bone growth (Figure 8).

Discussion

The usual processes required for producing hard tissues are biomineralization, extracellular matrix secretion, mesenchymal cell recruitment, proliferation, and differentiation.[6] Throughout the whole bone fracture healing process from the first stages of cartilaginous callus development and remodeling to the last stages of new bone filling the fracture gap, the extracellular matrix acts as a structural support, migratory cell anchor, cell differentiation signal, and regulator of cell proliferation.[7]

Bone injury and fracture responses, as well as bone formation and maintenance, depend on the protein phase of the extracellular matrix of bone.[8]

Each year, nearly 7 million people visit orthopedic surgeons in the USA for bone fracture repairs, and thus they are very common orthopedic treatments. In the majority of cases, bone functions and structures recover after treatment for fractures without scarring, but healing sometimes takes longer than expected or does not occur at all, which can lead to increased healthcare costs, additional surgeries, and a longer recovery period, particularly in older patients, and it can be associated with higher mortality rates.[9]

Histological and histomorphometric analysis

Promising signs of healing were observed in both the control and experimental groups, according to the overall histological findings. Bone deposition and remodeling occurred, but the rates and durations of these processes varied (see Figures 9–20).

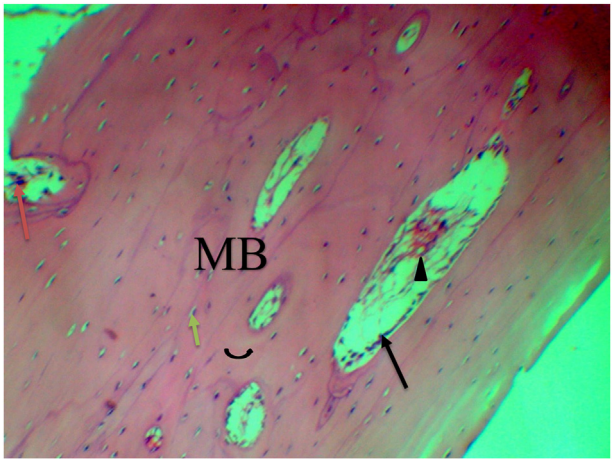

Figure 8: Histological image showing the presence of mature bone (MB), newly formed osteoblasts (black arrow), osteocytes (green arrow), osteoclasts (orange arrow) developing blood vessels (arrow head), and osteons (black ring) indicative of mature bone growth after the combined treatment for 14 days. H&E staining and 40× magnification.

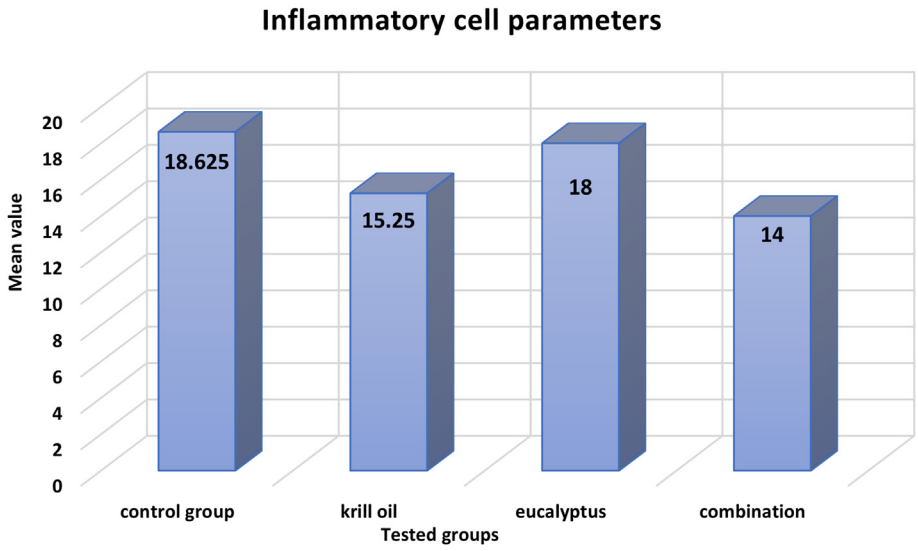

Figure 9: Differences in inflammatory cell parameters between groups after 7 days.

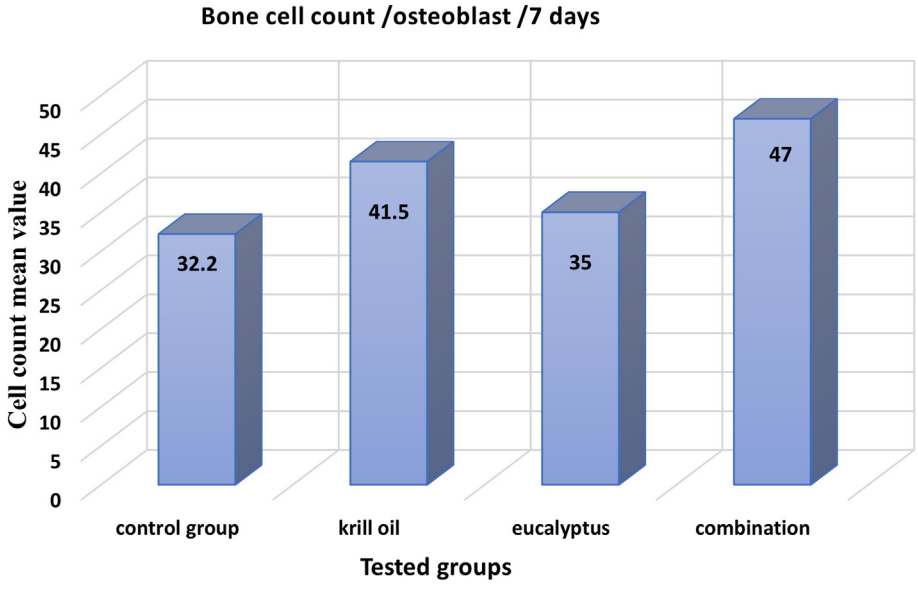

Figure 10: Differences in osteoblast cell counts between groups after 7 days.

Healing after 3 days

Examination of tissue samples showed that applying krill oil and eucalyptus oil, either alone or in combination, significantly decreased the number of inflammatory cells in the bone defect area in Wistar rats after 3 days compared with those that did not receive treatments. In particular, after treatment for 3 days, the lowest count of inflammatory cells was observed in the group that received a combination of krill oil and eucalyptus oil. The anti-inflammatory effect of krill oil may be due to its chemical composition, which primarily consists of omega-3 fatty acids.[10] Krill oil is made from krill and contains the same two types of fatty acids found in fish oil (*eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)*). However, krill contains higher concentrations of EPA and DHA, and much is bound to phospholipids, making it more bioavailable.[11] Thus, krill oil may mimic the benefits of fish oil while requiring only a fraction of the dosage.[12] Eucalyptus oil is rich in phytochemicals, such as tannins, alkaloids, flavonoids, and propanoids.[13] Around 20–80 chemicals have been obtained from various *Eucalyptus* species.[14]

Healing after 7 days

After 7 days, the deposition of osteoid bone was observed in bone sections in all groups. Immature bone spicules surrounded by osteoblasts were more evident in the experimental treatment groups (krill oil, eucalyptus oil, and combined treatment), which are consistent with previous findings, and they indicated accelerated osteoid matrix synthesis and mineralization through early cell differentiation.

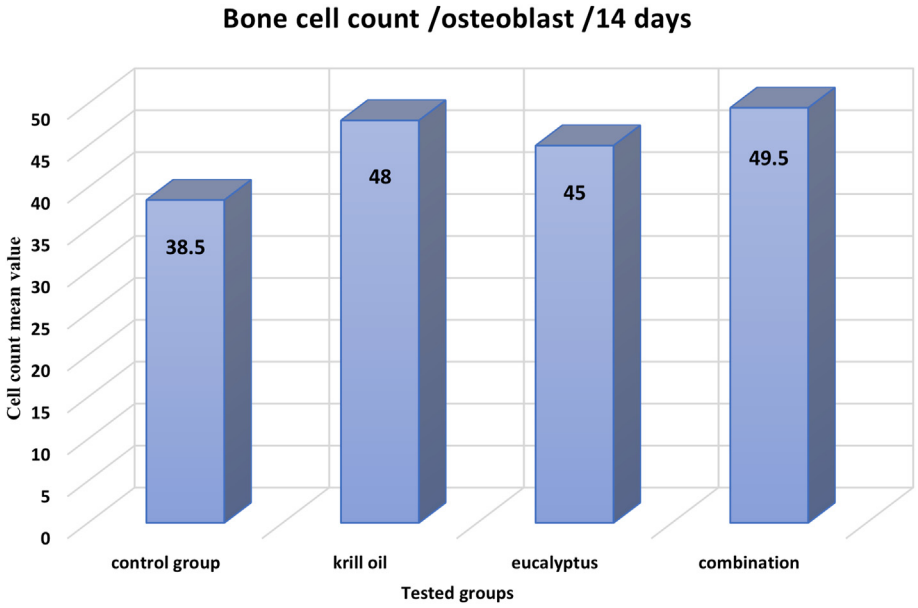

Figure 11: Differences in osteoblast cell counts between groups after 14 days.

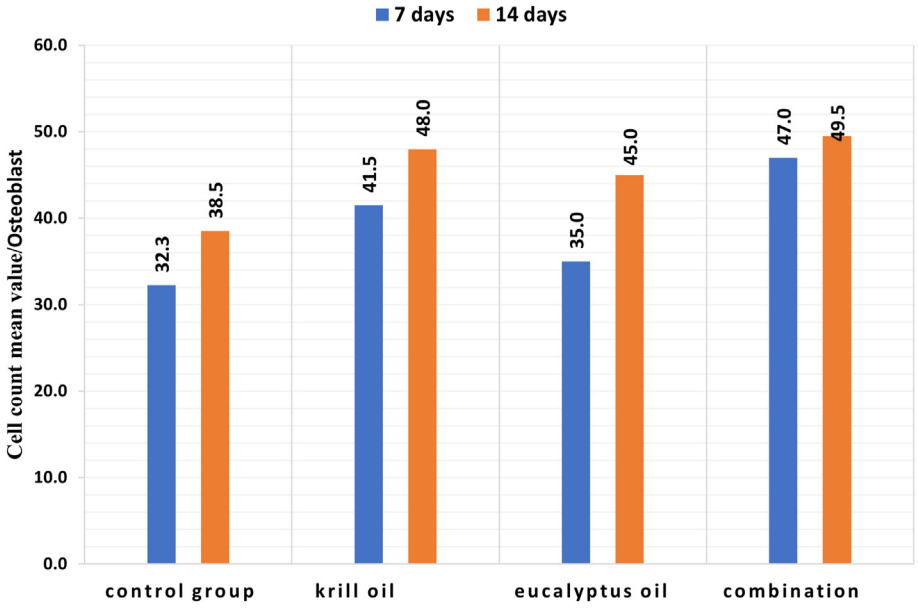

Figure 12: Comparison of osteoblast cell numbers after different periods.

Cellular proliferation peaked during the first week post-injury, followed by the maturation of cellular components and endochondral ossification toward the end of the second week. At 7 days after surgery, delicate and interlaced bone trabeculae filled the defect region according to observations of bone healing in the femurs of spontaneously hypertensive rats. In each specimen, cuboid osteoblasts encircled these newly formed trabeculae, which were characterized by numerous osteocytes.[15] The histological findings obtained in the current study after 7 days indicated matrix deposition filled with numerous large osteocytes surrounded by osteoblasts. Trabecular formation was not evident. Early osteoid deposition was observed, and significant increases were found in the average cell counts under the two individual treatments following recovery for 7 days. The effects of krill oil and eucalyptus oil did not differ significantly, with little difference in the mean cell counts. However, the most effective acceleration of healing was found under the combined treatment, especially in terms of the mean number of osteoblasts. The number of osteoblasts was significantly higher under the krill oil treatment compared with the eucalyptus oil treatment and control treatment according to microscopic analysis following therapy for 7 days. Similarly, a previous study[16] reported the presence of these structures and the temporary accumulation of young bone with low mineral density. Histological analysis of bone defects following treatment with krill oil for 7 days post-surgery demonstrated new

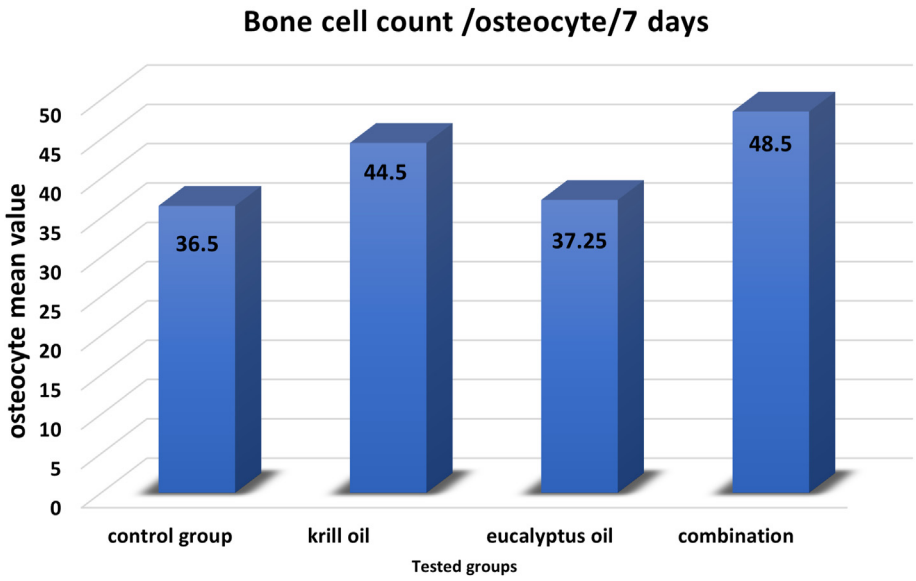

Figure 13: Differences in osteocyte cell counts after 7 days.

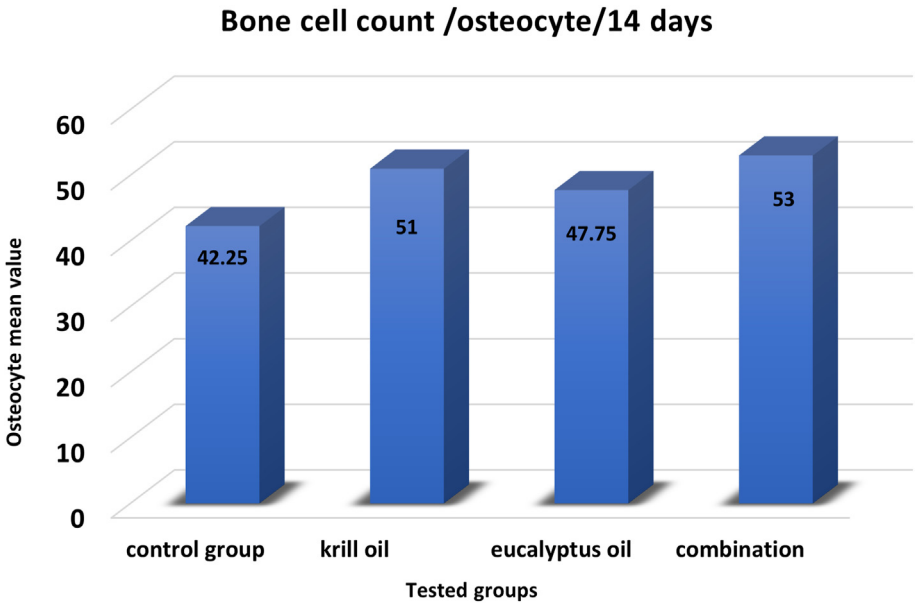

Figure 14: Differences in osteocyte cell counts after 14 days.

bone development, as shown by the increases in the osteoblast count and abundance of bone trabeculae compared with the control and eucalyptus oil treatment groups. Similar results were obtained in a previous study[17] based on microscopic examinations of histological sections after receiving a combination of krill oil and eucalyptus oil for 7 days following surgery. Under the combined treatment, increases were found in the numbers of osteoblasts and osteocytes, as well as decreases in the number of osteoclasts compared with the other treatments. In addition, the trabecular bone thickness was significantly higher and the bone marrow area was smaller compared with the other treatments. These differences can be attributed to the effects of the various chemical components of krill oil and eucalyptus oil, as well as their combined effect on progenitor cells, causing them to differentiate into osteoblasts (cells that form bone) and enhancing the formation of osteoid tissue due to the additional nutrient supplements in the combined treatment.

Healing after 14 days

In both the experimental and control treatment groups, irregular bone trabeculae formed, according to histological analyses. However, remodeling and bone deposition occurred at different rates. The newly formed bone trabeculae contained osteoclasts inside their lacunae and osteoblasts lined the borders of the trabeculae. Fibrovascular marrow was also observed between the newly formed trabeculae. These findings are consistent with those obtained in previous studies.[18,19] In

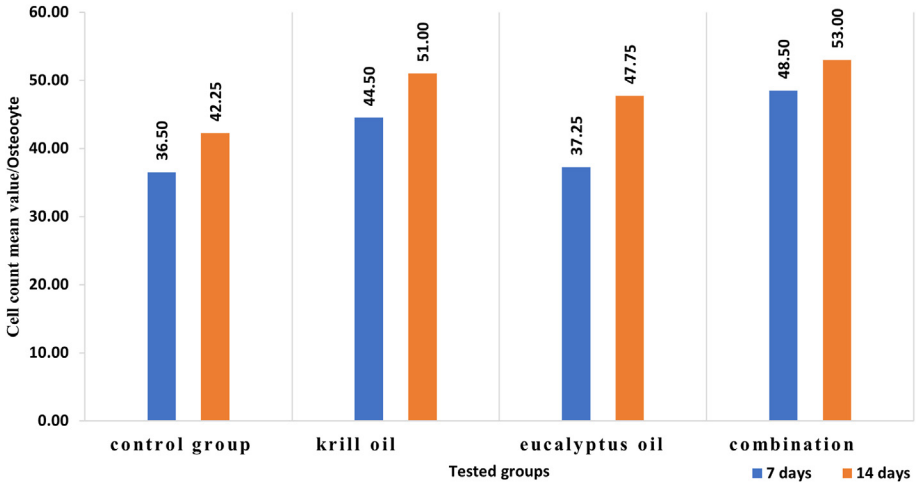

Figure 15: Differences in osteocyte cell counts after 7 and 14 days.

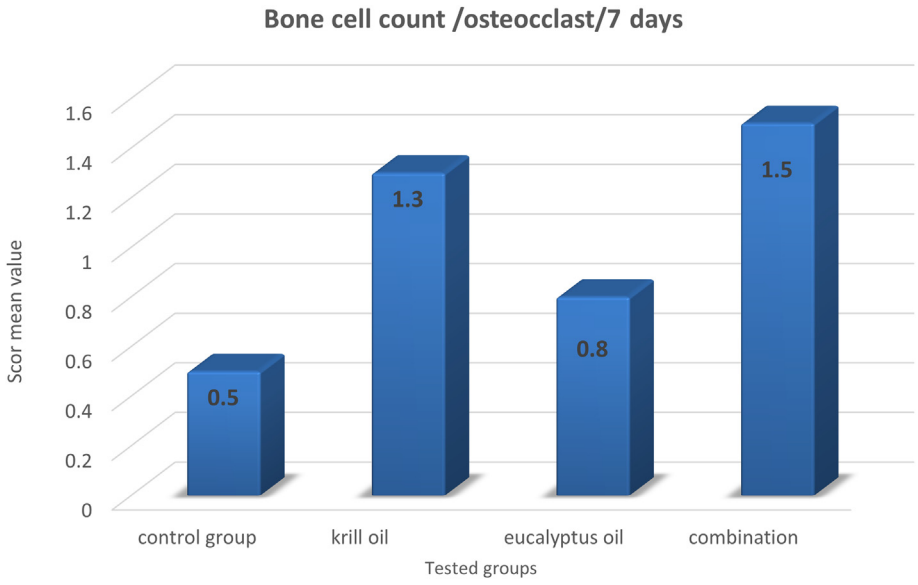

Figure 16: Differences in osteoclast cell counts after 7 days.

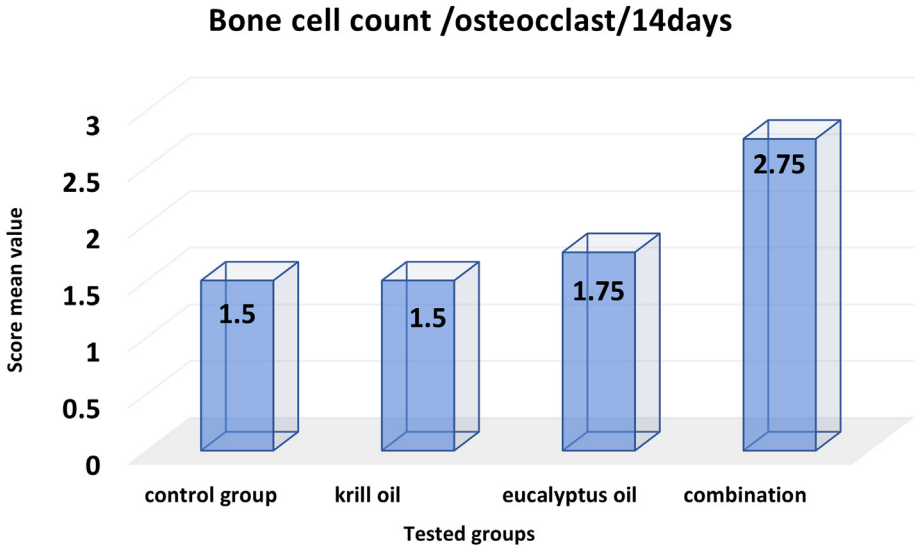

Figure 17: Differences in osteoclast cell counts after 14 days.

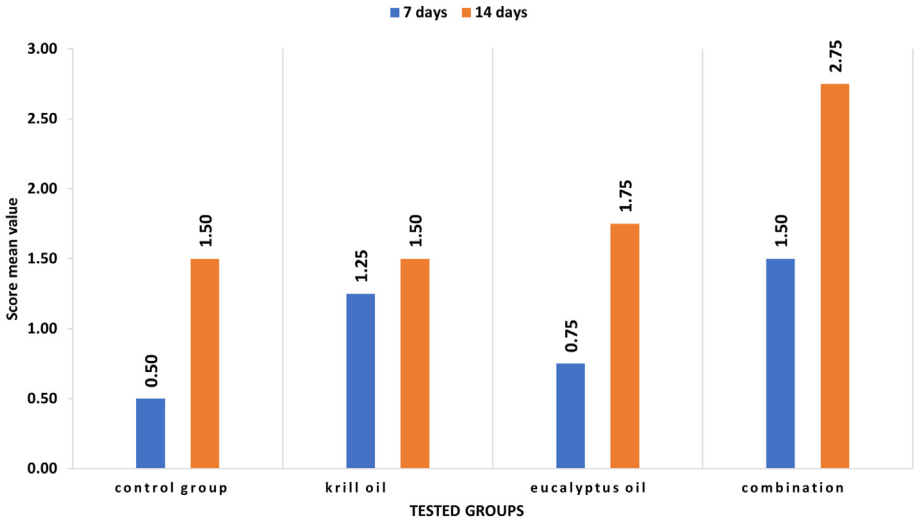

Figure 18: Comparison of osteoclast cell counts after different periods.

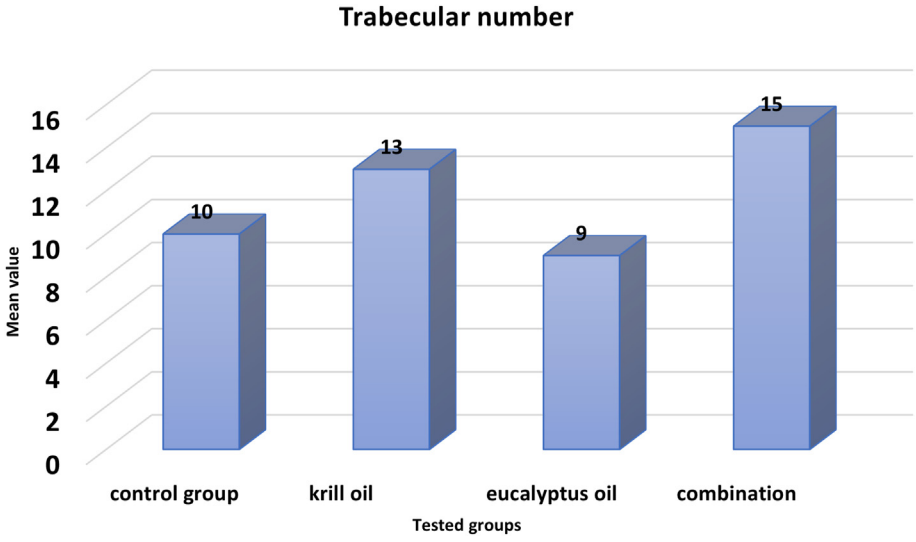

Figure 19: Differences in trabecular numbers after 14 days.

the present study, at 14 days after surgery, the defective area was filled with delicate and intertwined bone trabeculae. Numerous osteocytes were observed inside the freshly created trabeculae and they were encircled by cuboid osteoblasts in each case. The findings obtained with krill oil were consistent with those reported in previous studies.

After 14 days, microscopic examination of the bone defects indicated a higher osteoclastic activity level in the group treated using krill oil compared with the control. Similarly, a previous study[20] indicated that the stimulation and activation of osteoclast differentiation were essential for bone remodeling and repair. In the present study, the osteoclastic activity was higher in bone defects under krill oil treatment compared with the control and eucalyptus oil treatment groups, as also shown in a previous study at 14 days after forming drill-hole defects in the cortex,[21] as well as in another study.[22] In addition, histological analysis showed that treatment using a combination of krill oil and eucalyptus oil for 14 days after surgery led to a higher average number of osteocytes and lower average osteoblast count compared with the other treatments. The delay in bone apposition and maturation may have been due to the time-consuming process, and the reduction in the number of osteoblasts over time could be explained by the increased need for formative cells, supplements, and nourishment during tissue formation. After the bone reached its final size, fewer osteoblasts and blood vessels were needed, except for biological activity maintenance, as shown by the effect size coefficient.

The increases in the numbers of osteoblasts and osteocytes, and decreases in bone marrow spaces indicated bone healing maturation, and our results are consistent with previous findings.[23]

Bone marrow stromal cells

Bone marrow stromal cells are stem cells that can develop into different types of cells, such as hematopoietic,

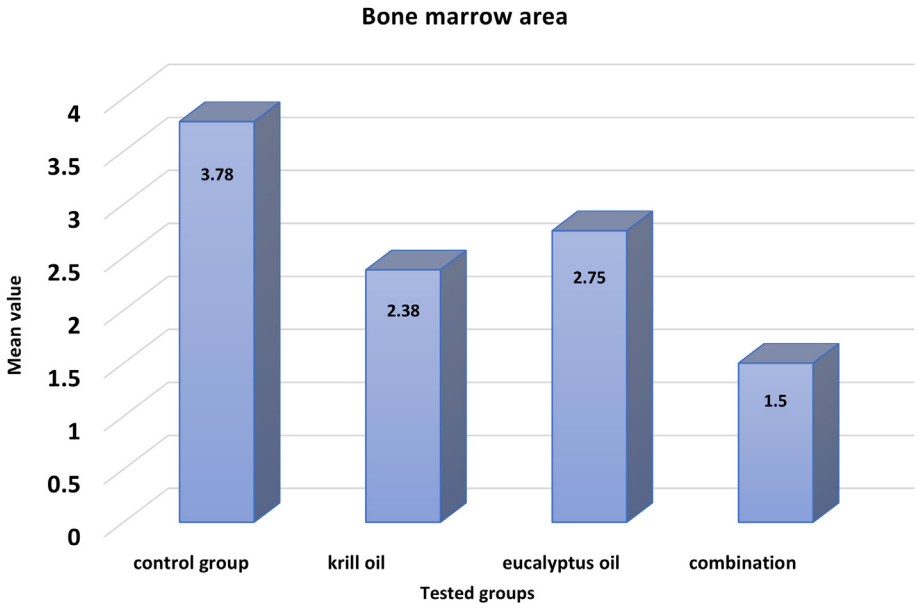

Figure 20: Differences in bone marrow areas after 14 days.

chondrogenic, or osteogenic cells. These cells can belong to various lineages, such as fibroblastic, reticular, adipogenic, and osteogenic cell lineages, and they have been extensively studied in both animal and clinical trials. Bone marrow stromal cells have a high capacity to multiply and they have been found to accelerate the repair of bone injuries. However, they do not fully mature into osteoblasts without outside triggers in laboratory settings.[24] Originally, it was considered that osteoblasts, the cells responsible for mineralizing the bone matrix, are mostly derived from osteoprogenitor cells.[25] Autologous progenitor cells are obtained from bone tissue and contribute to the regular remodeling and repair process, as indicated in previous research.[26] Subsequent research[27] suggests that mesenchymal cells play a role in the formation of the medullary callus during bone marrow regeneration, without the need for endochondral ossification.

Mesenchymal cells originating from bone marrow are involved in bone bridge construction, as demonstrated in a previous study.[28] The bone bridge is built successfully after 14 days and as it continues to mature in the following days, decreases occur in the populations of mesenchymal cells as well as the many basic cell types that make up the mesenchymal matrix. Osteoprogenitor cells generated from bone marrow are recruited to injury sites during intramembranous ossification.[29] Two types of stem cells collaborate to regulate the bone volume: cells derived from hematopoietic stem cells and mesenchymal stem cells, which become osteoblasts and osteoclasts, respectively. During the hard tissue development process, mesenchymal cells are recruited to proliferate, differentiate, secrete the extracellular matrix, and biomineralize.[30] The extracellular matrix is an essential signaling component with an essential role in the migration of cells, where it contains signals related to structural support, cell proliferation, and differentiation throughout each stage of the bone fracture healing process from the formation of a cartilaginous callus to callus remodeling and eventual bridging of the fracture gap.[31]

When a bone fractures, blood flows out because bones contain many arteries. A blood clot called a hematoma then develops around the fracture. This clot contains a protein meshwork that temporarily plugs the gap created by the break.[32] Next, the immune system is activated to regulate inflammation, an essential part of the healing process. The immune system sends signals to various organs, including blood and bone marrow, to attract stem cells to the fracture site, where they begin to produce cartilage and bone to facilitate the bone repair process.[33] Most fractures heal rapidly and without scarring, but the healing process might be affected by setbacks or have an unfavorable conclusion in some cases.[33] Older people may experience increased medical costs, additional surgeries, and prolonged recovery periods when fracture treatment is delayed or unsuccessful, ultimately leading to higher mortality rates.[34]

The general histological findings obtained in the present study indicated that every histological segment showed signs of effective healing in both the control and experimental treatment groups. Bones are deposited and remodeled at varying rates in different people. In the present study, after 14 days, the presence of newly formed bone trabeculae was observed in rats that received krill oil, eucalyptus oil, or a combination of both. Osteocytes were found inside these trabeculae, which were bordered by osteoblasts, as also found in previous research.[35] At 14 days after surgery, fragile and interlaced bone trabeculae were observed in the damaged area, with substantial amounts of osteocytes and cuboid osteoblasts present in all cases. Vascular endothelium and newly formed sensory and autonomic neurons are examples of mineralized tissues that are formed by osteoblasts during the many phases of bone production and healing.[36] The results can be explained by the eventual establishment of the majority of osteoblasts that reached the bone defect site leading to the formation

and maturation of woven bone. Most of the osteoblasts responsible for bone generation became trapped within the matrix and transformed into osteocytes, which eventually ceased secreting osteoid.[37] In a previous study[31] that involved cutting a hole in the center of the femur to induce a transcortical bone defect in mice, mature lamellar cortical bone filled the space after 4 weeks, and immature bone continued to collect in the drilled hole after 2 weeks. The repair of bone flaws and fracture is greatly dependent on neovascularization. In the present study, the freshly regenerated bone tissue contained a much lower total number of vessels in the treatment groups than the control group.

Our findings agree with those obtained in previous studies,[38,39] which observed a significant decrease in the trabecular number over time and after 14 days of treatment. In addition, significant decreases were found in the number of bone trabeculae around the Haversian canals and the bone marrow area under the combined treatment. Two weeks later, fresh bone had filled the hole, which indicated the accumulation of new bone in the drilled hole.

Conclusion

In the present study, osteogenesis and angiogenesis processes collaborated to create and restore bone tissue. The findings obtained in the present study suggest that applying a mixture of krill oil and eucalyptus oil may help to accelerate the recovery time for bone defects and help bone heal more effectively than each separate treatment.

Source of funding

Public, commercial, or non-profit funding entities did not provide any particular grant for this study.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

Ethical approval was granted by the University of Baghdad (Approval No. 583, 17/04/2022).

Authors contributions

EFK was responsible for the study's conception and design, as well as research, data collection, and organization. Data were examined and analyzed using SMS. As well as providing logistical help, EFK wrote the first and final drafts of the article. The content and similarity index of the text are the responsibility of all authors, who critically evaluated and approved the final draft.

Data availability statement

Data that support the findings obtained in this study are available from the corresponding author upon reasonable request.

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