

## Treatment with Carnitine Enhances Bone Fracture Healing under Osteoporotic and/or Inflammatory Conditions

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**Abstract:** The aim of this study was to examine the effects of carnitine on bone healing in ovariectomy (OVX) and inflammation (INF)-induced osteoporotic rats. The rats were randomly divided into nine groups (n = 8 animals per group): sham-operated (Group 1: SHAM); sham + magnesium silicate (Mg-silicate) (Group 2: SHAM + INF); ovariectomy (Group 3: OVX); ovariectomy + femoral fracture (Group 4: OVX + FRC); ovariectomy + femoral fracture + Mg-silicate (Group 5: OVX + FRC + INF); ovariectomy + femoral fracture + carnitine 50 mg/kg (Group 6: OVX + FRC + CAR50); ovariectomy + femoral fracture + carnitine 100 mg/kg (Group 7: OVX + FRC + CAR100); ovariectomy + femoral fracture + Mg-silicate + carnitine 50 mg/kg (Group 8: OVX + FRC + INF + CAR50); and ovariectomy + femoral fracture + Mg-silicate + carnitine 100 mg/kg (Group 9: OVX + FRC + INF + CAR100). Eight weeks after OVX, which allowed for osteoporosis to develop, INF was induced with subcutaneous Mg-silicate. On day 80, all of the rats in groups 4–9 underwent fracture operation on the right femur. Bone mineral density (BMD) showed statistically significant improvements in the treatment groups. The serum markers of bone turnover (osteocalcin and osteopontin) and pro-inflammatory cytokines (tumour necrosis factor  $\alpha$ , interleukin 1 $\beta$  and interleukin 6) were decreased in the treatment group. The X-ray images showed significantly increased callus formation and fracture healing in the groups treated with carnitine. The present results show that in a rat model with osteoporosis induced by ovariectomy and Mg-silicate, treatment with carnitine improves the healing of femur fractures.

Osteoporosis is a highly prevalent disease that is characterized by low bone density. Among various complications associated with osteoporosis, two of the most important are spontaneous fracture and increased fracture risk [1,2]. The most important parameter revealing the fracture risk is bone mineral density (BMD), as a decrease in BMD can significantly augment the risk of fracture [3]. Osteoporosis induced after ovariectomy (OVX) in rats is scientifically very close to senile osteoporosis occurring in human beings, and this model is frequently invoked experimentally [4,5]. In addition, the application of subcutaneous magnesium silicate (Mg-silicate) in rats subjected to OVX induces the acute phase response, and by contributing to the osteoporosis through inflammatory action, it leads to trabecular bone loss [6,7]. The post-menopausal interruption of oestrogen in women has an active role in many inflammatory processes, thereby increasing cytokine production around the bone. The most prevalent pro-inflammatory cytokines are interleukins (IL-1 $\beta$  and IL-6) and tumour necrosis factor (TNF)- $\alpha$ ; these corrupt bone turnover and lead to

bone resorption [8,9]. The osteoporosis model created with Mg-silicate induction after ovariectomy specifically reveals the role of oxidative stress and cytokines within the pathophysiology of the osteoporosis [10].

Cytokines increase depending on inflammation and oestrogen deficiency causing a delayed bone healing process. Thus, in this experimental study of fractures developing in the context of osteoporosis, we expect that the fractures will be aggravated in the inflammatory phase. In cases where delayed bone healing is evident, it is very important to study the bone status during the first month, which represents the retarded healing phases. Many anti-inflammatory and antioxidant substances have been experimentally studied, as in fractures occurring with osteoporosis, free oxygen radicals, inflammation and associated cytokines are all evident [11–14]. Carnitine plays a key role here. L-carnitine is a water-soluble molecule and has very important attributes related to mammalian metabolism, especially in the mitochondrial oxidation of normal fatty acid.

In our previous study, we showed that carnitine improved osteoporosis in osteoporotic rats and decreased the augmented cytokine levels due to osteoporosis [15]. In previous studies, anti-inflammatory effects of carnitine were shown in different experimental models [16,17]. In addition to its anti-inflammatory action, carnitine is a very powerful antioxidant [18–20]. Regarding our topic, it has been shown that carnitine decreases the increased oxidative stress in elderly people

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[21,22]. In another study that we carried out on carnitine, we demonstrated its important protective roles in neurotoxicity generated through bilirubin and kainic acid [23,24].

In the light of all this information, an experimental fracture model based on osteoporosis was created by means of OVX aggravated with Mg-silicate in rats. The effects of an anti-inflammatory antioxidant molecule 'carnitine' on bone healing were studied by means of BMD, radiography, pro-inflammatory cytokines, osteopontin and osteocalcin, which are important factors in bone healing.

### Materials and Methods

**Animals.** In this study, we used 72 female Albino Wistar rats (240–260 g), which were obtained from the Medical Experimental Research Center, Ataturk University (ATADEM). Animal experiments and procedures were performed in accordance with the national guidelines for the use and care of laboratory animals and were approved by Ataturk University's local animal care committee (Approval Number: 29.05.2009-6/83). Rats were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22°C under lighting controls (14-hr light/10-hr dark cycle). Standard rat chow and tap water were given *ad libitum*.

**Chemicals.** Carnitine was obtained from Life Time Nutritional Specialties, Inc. (Orange, CA, USA). Thiopental sodium was purchased from IE Ulagay A.S. (Istanbul, Turkey). Metamizole sodium (Novalgine 500 mg/ml injectable formulation) was obtained from Sanofi Aventis (Istanbul, Turkey), and all other chemicals for laboratory experimentation were purchased from Sigma Chemical Co. (St. Louis, USA) and Merck (Frankfurter, Germany).

**Experimental design.** All surgical procedures were performed under sterile operating conditions with the rats under general anaesthesia. Eight rats were sham-operated, while the 64 others underwent OVX operation under anaesthesia with an intraperitoneal injection of thiopental sodium injection (20 mg/kg). The rats were randomly divided into nine groups (n = 8): sham-operated (Group 1: SHAM); sham + Mg-silicate (Group 2: SHAM + INF); ovariectomy (Group 3: OVX); ovariectomy + femoral

fracture (Group 4: OVX + FRC); ovariectomy + femoral fracture + Mg-silicate (Group 5: OVX + FRC + INF); ovariectomy + femoral fracture + carnitine 50 mg/kg (Group 6: OVX + FRC + CAR50); ovariectomy + femoral fracture + carnitine 100 mg/kg (Group 7: OVX + FRC + CAR100); ovariectomy + femoral fracture + Mg-silicate + carnitine 50 mg/kg (Group 8: OVX + FRC + INF + CAR50); and ovariectomy + femoral fracture + Mg-silicate + carnitine 100 mg/kg (Group 9: OVX + FRC + INF + CAR100; table 1). After 2 months (8 weeks after ovariectomy), which allowed for osteoporosis to develop in the OVX rats, inflammation was induced in groups 2, 5, 8 and 9 through four separate subcutaneous injections of Mg-silicate (3.2 g total per animal) in sterile saline in the animals' backs.

After 20 days (on day 80, 20 days after the Mg-silicate administration), all of the rats in groups 4, 5, 6, 7, 8 and 9 underwent fracture operation and the right femur of all of the rats was fractured under anaesthesia. General anaesthesia for all operative procedures was achieved by intraperitoneal administration of 20 mg/kg sodium thiopental. Post-operative pain was controlled using a peritoneal injection of 150 mg/kg metamizole sodium (Novalgine) initially. Each animal's right hind limb was shaved, and then, a 2-cm lateral parapatellar incision was made, and the patella displaced laterally to expose the distal femoral condyle of the right hind limb as described before [25]. The femoral fracture model was established by cutting the femur transversely in the middle section. After manual reduction, the fractured femur was fixed with intramedullary Kirschner wires (diameter 1.0 mm, Shanghai Medical Apparatus Co. Ltd.-Shanghai, China). The wounds were then irrigated and closed using 4.0 nylon suture. The soft tissue and skin were closed with 4-0 Vicryl sutures. Group 1 was subjected to the same procedure except without cutting femur. Animals were allowed to drink and eat freely after the surgery.

On the first day after the fracture operation, carnitine intervention was started (on day 81 after beginning the study). Two different doses of carnitine (50 mg/kg and 100 mg/kg) were administered while the vehicle was given in the same volume to sham and control group rats (non-treated with carnitine). Carnitine was administered orally once daily for 30 days beginning on the post-operative day 1. All of the rats in groups 6 and 8 were given 50 mg/kg of carnitine for 30 days. All of the rats in groups 7 and 9 were administered 100 mg/kg of carnitine for 30 days. Thirty days after the fracture operation, rats were subjected to X-ray imaging after 20 mg/kg sodium thiopental anaes-

Table 1.

Experimental design.

GROUPS	1st day to 60th day	60th day	81st day	81st to 110th day	Killed 110th day
	Osteoporosis period	Inflammation induced by Mg-silicate	Fracture operation	Treatment period	
1 SHAM	Sham operation	–	–	–	i.p. thiopental sodium
2 SHAM + INF	Sham operation	Inflammation induced by Mg-silicate	–	–	
3 OVX	Ovariectomy operation	–	–	–	
4 OVX + FRC	Ovariectomy operation	–	Fracture operation	–	
5 OVX + INF + FRC	Ovariectomy operation	Inflammation induced by Mg-silicate	Fracture operation	–	
6 OVX + FRC + CAR50	Ovariectomy operation	–	Fracture operation	50 mg/kg CAR treatment	
7 OVX + FRC + CAR100	Ovariectomy operation	–	Fracture operation	100 mg/kg CAR treatment	
8 OVX + INF + FRC + CAR50	Ovariectomy operation	Inflammation induced by Mg-silicate	Fracture operation	50 mg/kg CAR treatment	
9 OVX + INF + FRC + CAR100	Ovariectomy operation	Inflammation induced by Mg-silicate	Fracture operation	100 mg/kg CAR treatment	

thetia and then killed for blood and tissue collection. Femurs were stored at  $-20^{\circ}\text{C}$  for biochemical testing.

**Dual-energy X-ray absorptiometry (DEXA) estimations.** The femur bones of the rats were evaluated *in vitro* after surgical removal. BMD was analysed by the DEXA method using a Discovery Wi (Hologic Inc., Bedford, MA, USA) equipped with the appropriate software for bone assessment in small animals. Same researcher performed each measurement, and all analyses were carried out using the same region of interest (ROI) window size.

**Serum measurements of osteocalcin, osteopontin, TNF- $\alpha$ , IL1- $\beta$  and IL-6.** Sera were separated from blood by allowing it to clot, followed by centrifugation at 3200 g for 10 min at  $4^{\circ}\text{C}$  and kept at  $-86^{\circ}\text{C}$  until thawed for the assay. The amounts of osteocalcin, osteopontin, TNF- $\alpha$ , IL1- $\beta$  and IL-6 in each sample were determined in duplicate with highly sensitive enzyme-linked immunosorbent assay (ELISA) kits specifically designed for rats (E90471Ra-Uscn Life Science Inc., E90899Ra-Uscn Life Science Inc., Invitrogen-KRC3011 [Burlington, USA], Invitrogen-KRC0011 [Massachusetts, USA] and RayBiotech-ELR-IL6.001 [Georgia, USA], respectively) according to the manufacturer's instructions.

**Statistical analysis.** Data on the serum cytokine levels were measured by ELISA and subjected to one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS version 19.0) software. Differences among the groups were identified using the Duncan's multiple range test option and were considered significant at  $p < 0.05$ . All data were expressed as mean  $\pm$  standard deviation (S.D.) in each group.

## Results

### Bone mineral density.

Femoral BMD ( $\text{g}/\text{cm}^2$ ) in the OVX group was markedly lower than that in SHAM group ( $0.211 \pm 0.018$  and  $0.257 \pm 0.020$ , respectively; table 2). Inflammation and fracture exacerbated this osteopenia in groups OVX + FRC and OVX + FRC + INF ( $0.199 \pm 0.020$  and  $0.200 \pm 0.011$ , respectively), whereas the SHAM groups showed no effect (SHAM + INF:  $0.255 \pm 0.013$ ). BMD showed statistically significant improvements in the treatment groups. Different doses of carnitine were able to restore BMD when compared to OVX, OVX + FRC and OVX + FRC + INF groups. BMD in the OVX + FRC + CAR50 ( $0.227 \pm 0.015$ ) and OVX + FRC

+ CAR100 ( $0.241 \pm 0.028$ ) was significantly higher than that in OVX + FRC ( $0.199 \pm 0.020$ ). In addition, BMD in OVX + FRC + INF + CAR50 ( $0.223 \pm 0.013$ ) and OVX + FRC + INF + CAR100 ( $0.233 \pm 0.006$ ) was significantly higher than that in OVX + FRC + INF ( $0.200 \pm 0.011$ ). The reduction in the BMD of the femur by fracture and inflammation was significantly prevented in all the treatment groups. Comparing the results for each therapy, the higher dose (100 mg/kg) of carnitine showed a greater increase in bone BMD levels than the lower dose of (50 mg/kg) carnitine. Both doses of carnitine resulted in decreased bone loss due to OVX and inflammation aggravated via Mg-silicate.

### Bone turnover.

As compared to sham, the serum markers of bone turnover (osteocalcin and osteopontin) significantly increased in groups OVX, OVX + FRC and OVX + FRC + INF (table 2). The osteopontin levels in the OVX, OVX + FRC and OVX + FRC + INF groups exposed to the Mg-silicate were significantly higher ( $19.20 \pm 2.42$ ,  $23.94 \pm 2.60$  and  $23.06 \pm 3.90$ , respectively) than those in SHAM group ( $14.32 \pm 3.39$ ). The osteocalcin levels in OVX, OVX + FRC and OVX + FRC + INF groups were also significantly higher ( $44.01 \pm 6.97$ ,  $49.05 \pm 6.53$  and  $44.90 \pm 5.68$ , respectively) than those in SHAM group ( $28.91 \pm 5.02$ ). Osteopontin and osteocalcin values were measured as  $21.05 \pm 2.92$  and  $40.92 \pm 2.69$  in OVX + FRC + CAR50 and  $17.88 \pm 2.43$  and  $37.33 \pm 4.04$  in OVX + FRC + CAR100, respectively.

After the administration of carnitine at 50 or 100 mg/kg, the levels of osteopontin and osteocalcin decreased to  $22.14 \pm 3.13$  and  $43.02 \pm 5.59$  and to  $19.16 \pm 1.77$  and  $39.39 \pm 2.34$  in OVX + FRC + INF + CAR50 and OVX + FRC + INF + CAR100, respectively. In addition, the increased level of the bone turnover markers in OVX, OVX + FRC and OVX + FRC + INF groups was recovered with carnitine administration.

### Markers of inflammation.

To determine the Mg-silicate-induced inflammation changes, we examined the pro-inflammatory cytokines in the serum using enzyme-linked immunoassay (ELISA) (table 3). TNF- $\alpha$ , IL1- $\beta$

Table 2.

Femoral BMD values and serum levels of osteopontin and osteocalcin.

	GROUPS	Osteopontin ng/ml	Osteocalcin ng/ml	BMD $\text{g}/\text{cm}^2$
1	SHAM	$14.32 \pm 3.39^{\text{a}}$	$28.91 \pm 5.02^{\text{a}}$	$0.257 \pm 0.020^{\text{d}}$
2	SHAM + INF	$15.66 \pm 2.66^{\text{a,b}}$	$31.71 \pm 2.53^{\text{a}}$	$0.255 \pm 0.013^{\text{d}}$
3	OVX	$19.20 \pm 2.42^{\text{c,d}}$	$44.01 \pm 6.97^{\text{c,d}}$	$0.211 \pm 0.018^{\text{a,b}}$
4	OVX + FRC	$23.94 \pm 2.60^{\text{c}}$	$49.05 \pm 6.53^{\text{c}}$	$0.199 \pm 0.020^{\text{a}}$
5	OVX + INF + FRC	$23.06 \pm 3.90^{\text{c}}$	$44.90 \pm 5.68^{\text{d,e}}$	$0.200 \pm 0.011^{\text{a}}$
6	OVX + FRC + CAR50	$21.05 \pm 2.92^{\text{d,e}}$	$40.92 \pm 2.69^{\text{b,c,d}}$	$0.227 \pm 0.015^{\text{b,c}}$
7	OVX + FRC + CAR100	$17.88 \pm 2.43^{\text{b,c}}$	$37.33 \pm 4.04^{\text{b}}$	$0.241 \pm 0.028^{\text{c,d}}$
8	OVX + INF + FRC + CAR50	$22.14 \pm 3.13^{\text{d,e}}$	$43.02 \pm 5.59^{\text{c,d}}$	$0.223 \pm 0.013^{\text{b,c}}$
9	OVX + INF + FRC + CAR100	$19.16 \pm 1.77^{\text{c,d}}$	$39.39 \pm 2.34^{\text{b,c}}$	$0.233 \pm 0.006^{\text{c,d}}$

For each parameter, means that have same letter are not significantly different from the test of Duncan ( $p = 0.05$ ); namely, osteopontin sham is different from OVX group but similar with SHAM + INF group. Results are means  $\pm$  S.D., OVX, ovariectomy, OVX + FRC: ovariectomy + femoral fracture; OVX + INF + FRC, ovariectomy + fracture + Mg-silicate; CAR, carnitine.

Table 3.

Serum levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6.

GROUPS		TNF- $\alpha$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-6 (pg/ml)
1	SHAM	37.68 $\pm$ 3.75 <sup>a</sup>	55.45 $\pm$ 7.71 <sup>a</sup>	104.20 $\pm$ 12.13 <sup>a</sup>
2	SHAM + INF	55.39 $\pm$ 11.30 <sup>b,c,d</sup>	70.32 $\pm$ 8.47 <sup>a,b</sup>	150.10 $\pm$ 24.79 <sup>a</sup>
3	OVX	60.63 $\pm$ 7.69 <sup>d</sup>	80.90 $\pm$ 16.92 <sup>b,c</sup>	291.39 $\pm$ 39.28 <sup>c</sup>
4	OVX + FRC	63.09 $\pm$ 10.10 <sup>d,e</sup>	97.78 $\pm$ 10.74 <sup>d,e</sup>	409.13 $\pm$ 50.90 <sup>d,e</sup>
5	OVX + INF + FRC	71.96 $\pm$ 9.95 <sup>e</sup>	103.42 $\pm$ 18.40 <sup>e</sup>	465.17 $\pm$ 68.74 <sup>f</sup>
6	OVX + FRC + CAR50	58.29 $\pm$ 8.33 <sup>c,d</sup>	85.18 $\pm$ 20.05 <sup>c,d</sup>	375.52 $\pm$ 50.48 <sup>d</sup>
7	OVX + FRC + CAR100	47.32 $\pm$ 8.36 <sup>b</sup>	71.22 $\pm$ 10.62 <sup>b,c</sup>	243.84 $\pm$ 16.93 <sup>b</sup>
8	OVX + INF + FRC + CAR50	57.17 $\pm$ 11.03 <sup>c,d</sup>	84.31 $\pm$ 8.33 <sup>c,d</sup>	449.40 $\pm$ 80.74 <sup>e,f</sup>
9	OVX + INF + FRC + CAR100	50.16 $\pm$ 8.69 <sup>b,c</sup>	78.87 $\pm$ 13.68 <sup>b,c</sup>	308.56 $\pm$ 37.32 <sup>c</sup>

For each parameter, means that have same letter are not significantly different to the test of Duncan ( $p = 0.05$ ); namely, TNF- $\alpha$  OVX is different from sham group but similar to OVX + FRC group and CAR50-treated groups. Results are means  $\pm$  S.D., OVX, ovariectomy; OVX + FRC, ovariectomy + fracture; OVX + INF + FRC, ovariectomy + fracture + Mg-silicate; CAR, carnitine.

and IL-6 serum levels significantly elevated in OVX + FRC (63.09  $\pm$  10.10, 97.78  $\pm$  10.74 and 409.13  $\pm$  50.90, respectively) and OVX + FRC + INF (71.96  $\pm$  9.95, 103.42  $\pm$  18.40 and 465.17  $\pm$  68.74, respectively) in comparison with SHAM (37.68  $\pm$  3.75, 55.45  $\pm$  7.71 and 104.20  $\pm$  12.13, respectively).

The observed elevations of TNF- $\alpha$ , IL1- $\beta$  and IL-6 in the sera of OVX + FRC decreased significantly to 58.29  $\pm$  8.33, 85.18  $\pm$  20.05 and 375.52  $\pm$  50.48, respectively, in OVX + FRC + CAR50 and 47.32  $\pm$  8.36, 71.22  $\pm$  10.62 and 243.84  $\pm$  16.93, respectively, in OVX + FRC + CAR100. The elevations of TNF- $\alpha$ , IL1- $\beta$  and IL-6 in the sera of the rat groups that underwent OVX, fracture and Mg-silicate administration decreased significantly to 57.17  $\pm$  11.03, 84.31  $\pm$  8.33 and 449.40  $\pm$  80.74, respectively, in OVX + FRC + INF + CAR50 and 50.16  $\pm$  8.69, 78.87  $\pm$  13.68 and 308.56  $\pm$  37.32, respectively, in OVX + FRC + INF + CAR100. Moreover, both carnitine treatments decreased these serum cytokine levels in both the OVX and inflammation-induced fracture groups.

#### X-ray imaging.

X-ray images of the healed fractured femurs were evaluated to determine the stages of fracture healing and callus formation. The staging was done according to a 5-point scoring system (table 4) [26]. A comparison of the X-ray images of OVX + FRC (fig. 1A) and OVX + FRC + INF (fig. 1B) revealed that the administration of Mg-silicate resulted in minimal callus formation, intense inflammation and compromised fracture healing. For fracture healing staging, OVX + FRC + CAR50 had an average score of 4 (fig. 2A), OVX + FRC + CAR100 had a score of 4 (fig. 2B), OVX + FRC + INF + CAR50 had a score above 3 (fig. 2C), and OVX + FRC + INF + CAR100 had a score above 3 (fig. 2D). The average score of the OVX + FRC group was 2.17  $\pm$  0.41 and OVX + FRC + INF group was 1.67  $\pm$  0.52, indicating that the fracture healing was insufficient; the scores of carnitine 50 and 100 mg/kg groups without Mg-silicate administration were 3.67  $\pm$  0.52 and 3.83  $\pm$  0.41, respectively ( $p < 0.05$  for both), indicating that fracture union had occurred. The average scores of the groups administered both carnitine and Mg-silicate (OVX + FRC + INF + CAR50: 3.17  $\pm$  0.41 and OVX + FRC + INF + CAR100: 3.33  $\pm$  0.52) were also higher

Table 4.

The 5-point radiographic scoring system (modified from Warden *et al.* [26]) used to determine (A) the fracture healing stage and (B) the callus stage.

Score	Description
(A)	
0	No evidence of healing
1	Callus formation evident but fracture gap not bridged
2	Callus formation evident with bridging of the fracture gap but fracture line evident
3	Callus formation evident with bridging of the fracture gap with only faint fracture line
4	Fracture union
(B)	
0	No callus
1	Callus + (very minimal callus)
2	Callus ++ (minimal callus)
3	Callus +++ (moderate callus)
4	Callus ++++ (exuberant callus)

than that of OVX + FRC + INF (1.68  $\pm$  0.52) group ( $p < 0.05$  for both). All results show that both doses of carnitine produced sufficient fracture union process in both OVX + FRC and OVX + FRC + INF applications. The callus staging results were consistent with fracture healing staging, revealing that the control group exhibited significantly lower stages of callus formation than all of the carnitine groups.

## Discussion

In this study, the effects of carnitine on bone fracture healing in ovariectomized rats whose osteoporosis were aggravated with Mg-silicate were studied. Our results showed that carnitine has a potentiating effect on bone healing which was evaluated by several biochemical (serum cytokine, osteocalcin and osteopontin levels) and radiological parameters (BMD and X-ray).

The results of our study revealed that, without carnitine treatment, OVX increased the amounts of osteopontin and osteocalcin, leading to a negative alteration in bone turnover. Our BMD and X-ray results also supported this claim. When fracture was added to OVX, the osteopontin and osteocalcin

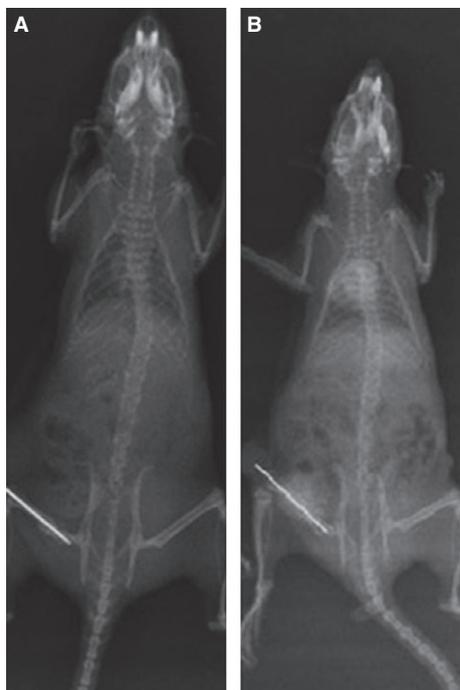


Fig. 1. X-ray graphics of rats from: OVX + FRC group, moderate callus formation and bridging of the fracture gap with faint fracture line (mean score:  $2.17 \pm 0.4$ ) (A); OVX + FRC + INF group, minimal callus formation with inflammatory response around the fracture line (mean score:  $1.67 \pm 0.52$ ) (B).

levels increased and the BMD index decreased. Combining the chronic inflammation 'by means of Mg-silicate administration' with OVX did not change the osteopontin, osteocalcin and BMD values in the fracture groups. Through carnitine administration in both fractures induced in osteoporotic rats

and in osteoporotic rats with chronic inflammation, significant healing was demonstrated in terms of the osteopontin and osteocalcin levels, which play a role in the pro-inflammatory cytokines and bone healing. This clearly shows that carnitine administration accelerated the healing of fractures induced in osteoporotic rats. Osteopontin, which is a multifunctional glycoprotein produced from mineralized tissue [27], decreases osteoclastic bone resorption and osteoblastic bone formation [28]. Previous studies have discussed the biphasic effect of osteopontin and reported that it is responsible for the proliferation and differentiation of osteoblastic cells during the bone creation [29,30]. If we focus on osteopontin in the context of osteoporosis, we may say that it increases with osteoporosis and this increases the points of osteoclastic activation [31]. In our study, as in previous research, the osteopontin level increased in osteoporotic rats; when fracture was induced in the osteoporotic rats, this effect was heightened. Carnitine treatment decreased this increased osteopontin level. This may be responsible for accelerating the bone healing, as our BMD results showed that carnitine administration increased the BMD levels. Thus, we consider that an increased BMD level might develop indirectly due to decreased osteoclastic activity.

Osteocalcin is also an important matrix protein produced from osteoblasts and is found dispersed in the cortical and trabecular bone [32]. It is considered that osteocalcin has important roles in the early-phase of bone healing [33] and contributes to the regulation of osteoblastic activity [33]. Studies have also demonstrated that during the early healing of the bone, osteocalcin is chemotactic for the osteoclastic cells [34]. In osteoporosis, osteocalcin is secreted by osteoblasts and is responsible for bone mineralization and calcium ion homeostasis [35]. Kim *et al.* [36] reported that serum osteocalcin levels

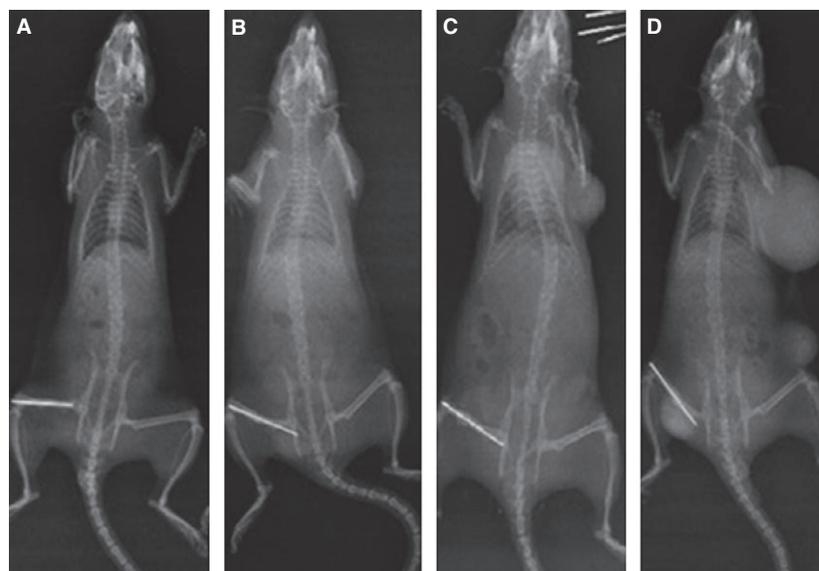


Fig. 2. X-ray graphics of rats from groups 1–4: exuberant callus formation in OVX + FRC + CAR50 group, fracture union (mean score:  $3.67 \pm 0.52$ ) (A); exuberant callus formation in OVX + FRC + CAR100 group, fracture union (mean score:  $1.83 \pm 0.41$ ) (B); exuberant callus formation in OVX + FRC + INF + CAR50 group, fracture union (mean score:  $3.17 \pm 0.41$ ) (C); and exuberant callus formation in OVX + FRC + INF + CAR100 group, fracture union (mean score:  $3.33 \pm 0.52$ ) (D).

increase in the context of osteoporosis. In our study, the osteocalcin levels were found to be high in osteoporotic rats, and these levels were even higher in osteoporotic rats with induced fractures. Carnitine administration decreased the increased osteocalcin levels. This decrease was found to be inversely correlated with BMD levels.

As another parameter during bone healing in osteoporotic rats with chronic inflammation, we studied TNF- $\alpha$ , IL-1 $\beta$  and IL-6 values. We saw that OVX alone increased these cytokines, and when the fracture occurred in addition to chronic inflammation, cytokine release reached peak levels of our study. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are among the important cytokines for bone healing. Bone fracture healing occurs in three phases, and in the context of osteoporosis, these phases are prolonged. The prolongation of the inflammatory phase in the first phase of bone healing seems to delay the fracture healing [37]. Failure to eliminate these pro-inflammatory cytokines when necessary leads to a delay in the fracture healing. Furthermore, the inflammation induced by Mg-silicate leads to an additional increase in these cytokines and prevents the later phases of bone healing. We have conducted many previous studies in this field and shown that pro-inflammatory cytokine levels are augmented during osteoporosis [38]. At the same time, these cytokines lead to an increase in oxygen radicals. Scientific studies proved that the increased free oxygen radicals decrease osteoblastic activity and increase osteoclastic activity [39–41]. At the same time, these cytokines have been reported to play a role in the synthesis of the extracellular matrix and stimulation of angiogenesis [42,43]. The levels of these cytokines increase after fracture but approach normal values after the first week [37]. In our study, this increasing cytokine amount was found to be higher in the control group, even at the end of 1 month; thus, we may say that the delayed healing of fractures is associated with the increased level of cytokines in the context of osteoporosis. Carnitine might trigger this effect by its anti-osteoporotic effect or directly in fracture healing. In one of our previous studies, we showed that administration of carnitine creates an anti-osteoporotic effect and decreases the cytokine level [15]. Other studies carried out on carnitine reported that it has a strong anti-inflammatory effect [16,17]. In addition to its anti-inflammatory role, carnitine also acts as a very powerful antioxidant. *In vivo* and *in vitro* studies have shown that carnitine has antioxidant effects in many cells [18–20].

As a result of our study, we may state that carnitine administration for the healing of rat femur fractures created in the context of osteoporosis induced with 'OVX and Mg-silicate' accelerated the healing of bone fractures. Moreover, it corrected the increase in osteopontin and osteocalcin values depending on either osteoporosis or fracture, and at the same time, it decreased the levels of pro-inflammatory cytokines. This effect might have occurred due to the anti-osteoporotic, anti-inflammatory, anticytokine and antioxidant properties of carnitine. This pre-study will lead to the usage of carnitine in either osteoporosis or fracture healing as a supplement. Its use will be supported with clinical studies in future.

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#### Conflict of Interest

The authors declare that there is no conflict of interest.

#### References

- Alexeeva L, Burkhardt P, Christiansen C, Cooper C, Delmas P, Johnell O *et al.* Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser 1994;**843**:1–129.
- Dogan A, Demirci S, Bayir Y, Halici Z, Karakus E, Aydin A *et al.* Boron containing poly-(lactide-co-glycolide) (PLGA) scaffolds for bone tissue engineering. Mater Sci Eng C Mater Biol Appl 2014;**44**:246–53.
- Nguyen T, Sambrook P, Kelly P, Jones G, Lord S, Freund J *et al.* Prediction of osteoporotic fractures by postural instability and bone density. BMJ 1993;**307**:1111–15.
- Namkung-Matthai H, Appleyard R, Jansen J, Hao Lin J, Maas-tricht S, Swain M *et al.* Osteoporosis influences the early period of fracture healing in a rat osteoporotic model. Bone 2001;**28**:80–6.
- Halici Z, Borekci B, Ozdemir Y, Cadirci E, Suleyman H. Protective effects of amlodipine and lacidipine on ovariectomy-induced bone loss in rats. Eur J Pharmacol 2008;**579**:241–5.
- Dinarello CA. Interleukin-1 and the pathogenesis of the acute-phase response. N Engl J Med 1984;**311**:1413–18.
- Pfeilschifter J, Wuster C, Vogel M, Enderes B, Ziegler R, Minne HW. Inflammation-mediated osteopenia (Imo) during acute-inflammation in rats is due to a transient inhibition of bone-formation. Calcif Tissue Int 1987;**41**:321–5.
- Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD. Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells *in vitro*. J Bone Miner Res 1989;**4**:113–18.
- Pacifici R. Cytokines and osteoclast activity. Calcif Tissue Int 1995;**56**(Suppl 1):S27–8.
- Minne HW, Pfeilschifter J, Scharla S, Mutschelknauss S, Schwarz A, Krempien B *et al.* Inflammation-mediated osteopenia in the rat: a new animal model for pathological loss of bone mass. Endocrinology 1984;**115**:50–4.
- Mohamad S, Shuid AN, Mohamed N, Fadzilah FM, Mokhtar SA, Abdullah S *et al.* The effects of alpha-tocopherol supplementation on fracture healing in a postmenopausal osteoporotic rat model. Clinics (Sao Paulo) 2012;**67**:1077–85.
- Mohd Ramli ES, Suhaimi F, Ahmad F, Shuid AN, Mohamad N, Ima-Nirwana S. Piper sarmentosum: a new hope for the treatment of osteoporosis. Curr Drug Targets 2013;**14**:1675–82.
- Liang W, Luo Z, Ge S, Li M, Du J, Yang M *et al.* Oral administration of quercetin inhibits bone loss in rat model of diabetic osteopenia. Eur J Pharmacol 2011;**670**:317–24.
- Aydin A, Halici Z, Akoz A, Karaman A, Ferah I, Bayir Y *et al.* Treatment with alpha-lipoic acid enhances the bone healing after femoral fracture model of rats. Naunyn Schmiedebergs Arch Pharmacol 2014;**387**:1025–36.
- Orsal E, Halici Z, Bayir Y, Cadirci E, Bilen H, Ferah I *et al.* The role of carnitine on ovariectomy and inflammation-induced osteoporosis in rats. Exp Biol Med (Maywood) 2013;**238**:1406–12.
- Caruso A, Cutuli VM, De Bernardis E, Leonardi G, Amico-Roxas M. Protective effect of propionyl-L-carnitine against PAF-induced rat paw oedema. Pharmacol Res 1995;**31**:67–72.
- Izgun-Uysal VN, Agac A, Derin N. Effect of L-carnitine on carrageenan-induced inflammation in aged rats. Gerontology 2003;**49**:287–92.

- 18 Gulcin I. Antioxidant and antiradical activities of L-carnitine. *Life Sci* 2006;**78**:803–11.
- 19 Jing L, Zhou LJ, Li WM, Zhang FM, Yuan L, Li S *et al*. Carnitine regulates myocardial metabolism by Peroxisome Proliferator-Activated Receptor- $\alpha$  (PPAR $\alpha$ ) in alcoholic cardiomyopathy. *Med Sci Monit* 2011;**17**:BR1–9.
- 20 Augustyniak A, Skrzydlewska E. The influence of L-carnitine supplementation on the antioxidative abilities of serum and the central nervous system of ethanol-induced rats. *Metab Brain Dis* 2010;**25**:381–9.
- 21 Rosca MG, Lemieux H, Hoppel CL. Mitochondria in the elderly: is acetylcarnitine a rejuvenator? *Adv Drug Deliv Rev* 2009;**61**:1332–42.
- 22 Sethumadhavan S, Chinnakannu P. L-carnitine and alpha-lipoic acid improve age-associated decline in mitochondrial respiratory chain activity of rat heart muscle. *J Gerontol A Biol Sci Med Sci* 2006;**61**:650–9.
- 23 Tastekin A, Gepdiremen A, Ors R, Buyukokuroglu ME, Halici Z. Protective effect of L-carnitine against bilirubin-induced neuronal cell death. *Brain Dev* 2006;**28**:436–9.
- 24 Tastekin A, Gepdiremen A, Ors R, Emin Buyukokuroglu M, Halici Z. L-carnitine protects against glutamate- and kainic acid-induced neurotoxicity in cerebellar granular cell culture of rats. *Brain Dev* 2005;**27**:570–3.
- 25 Aydin A, Halici Z, Akpınar E, Aksakal AM, Saritemur M, Yayla M *et al*. What is the role of bosentan in healing of femur fractures in a rat model? *J Bone Miner Metab* 2014. DOI 10.1007/s00774-014-0622-6.
- 26 Warden SJ, Komatsu DE, Rydberg J, Bond JL, Hassett SM. Recombinant human parathyroid hormone (PTH 1-34) and low-intensity pulsed ultrasound have contrasting additive effects during fracture healing. *Bone* 2009;**44**:485–94.
- 27 Mckee MD, Nanci A. Osteopontin and the bone remodeling sequence - colloidal-gold immunocytochemistry of an interfacial extracellular-matrix protein. *Ann N Y Acad Sci* 1995;**760**:177–89.
- 28 Ishijima M, Rittling SR, Yamashita T, Tsuji K, Kurosawa H, Nifuji A *et al*. Enhancement of osteoclastic bone resorption and suppression of osteoblastic bone formation in response to reduced mechanical stress do not occur in the absence of osteopontin. *J Exp Med* 2001;**193**:399–404.
- 29 Aubin JE, Liu F, Malaval L, Gupta AK. Osteoblast and chondroblast differentiation. *Bone* 1995;**17**:77S–83S.
- 30 Stein GS, Lian JB. Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev* 1993;**14**:424–42.
- 31 Altintas A, Saruhan-Direskeneli G, Benbir G, Demir M, Purisa S. The role of osteopontin: a shared pathway in the pathogenesis of multiple sclerosis and osteoporosis? *J Neurol Sci* 2009;**276**:41–4.
- 32 Ingram RT, Clarke BL, Fisher LW, Fitzpatrick LA. Distribution of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. *J Bone Miner Res* 1993;**8**:1019–29.
- 33 Hauschka PV, Wians FH Jr. Osteocalcin-hydroxyapatite interaction in the extracellular organic matrix of bone. *Anat Rec* 1989;**224**:180–8.
- 34 Glowacki J, Lian JB. Impaired recruitment and differentiation of osteoclast progenitors by osteocalcin-deplete bone implants. *Cell Differ* 1987;**21**:247–54.
- 35 Goldstone AP, Howard JK, Lord GM, Ghatei MA, Gardiner JV, Wang ZL *et al*. Leptin prevents the fall in plasma osteocalcin during starvation in male mice. *Biochem Biophys Res Commun* 2002;**295**:475–81.
- 36 Kim SW, Park DJ, Park KS, Kim SY, Cho BY, Lee HK *et al*. Early changes in biochemical markers of bone turnover predict bone mineral density response to antiresorptive therapy in Korean postmenopausal women with osteoporosis. *Endocr J* 2005;**52**:667–74.
- 37 Mountziaris PM, Spicer PP, Kasper FK, Mikos AG. Harnessing and modulating inflammation in strategies for bone regeneration. *Tissue Eng Part B Rev* 2011;**17**:393–402.
- 38 Avsar U, Karakus E, Halici Z, Bayir Y, Bilen H, Aydin A *et al*. Prevention of bone loss by Panax ginseng in a rat model of inflammation-induced bone loss. *Cell Mol Biol (Noisy-le-grand)* 2013;**59** (Suppl):OL1835–41.
- 39 Das UN. Interaction(S) between essential fatty-acids, eicosanoids, cytokines, growth-factors and free-radicals - relevance to new therapeutic strategies in rheumatoid-arthritis and other collagen vascular diseases. *Prostaglandins Leukot Essent Fatty Acids* 1991;**44**:201–10.
- 40 Mody N, Parhami F, Sarafian TA, Demer LL. Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Biol Med* 2001;**31**:509–19.
- 41 Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. *Prog Lipid Res* 2001;**40**:125–48.
- 42 Marzona L, Pavolini B. Play and players in bone fracture healing match. *Clin Cases Miner Bone Metab* 2009;**6**:159–62.
- 43 Cho T-J GL, Barnes GL, Einborn TA. Cytokines and fracture healing. *Curr Opin Orthop* 2001;**12**:403–8.