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Evaluation of antioxidant and osteoprotective effects of silymarin in fracture healing

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Therapeutic or supportive agents are needed to ensure faster recovery of bone function in fracture injuries. Accordingly, this study aimed to evaluate the accelerating effects of silymarin on fracture healing through antioxidant balance mechanisms. In the study, 48 male rats were divided into three equal groups: control, sham, and silymarin. Rats with right tibia fractures were administered 50 mg/kg/day of silymarin and/or normal saline by gavage for 21 days. After administration, serum samples were analyzed for antioxidant-oxidant markers such as superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels. In addition, bone tissue was examined radiologically and histopathologically. Following silymarin administration, CAT and SOD enzyme activities increased compared to the control group, but the MDA level, which is an indicator of oxidative stress, decreased. Histopathologic and radiologic examination revealed that silymarin increased osteoblastic activity, stimulated bone formation, and exhibited osteoprotective effects. Silymarin may stimulate bone formation through oxidative stress inhibition and antioxidant effects in fracture injuries. It supports osteoprotective effects in wound healing mechanisms activated after fracture injury.

Keywords: Milk thistle supplement, Bone regeneration, Reactive oxygen species, Antioxidant defence, Osteogenic effect

Fracture healing is a complex multifactorial biological process involving the lymphatic and nervous system, capillaries, supporting cells, and osteoblastic and osteoclastic activities[1,2]. A series of coordinated mechanisms, such as inflammation, cellular proliferation, and maturation, are activated to restore disrupted bone integrity[3,4]. In the early phase of this process, the initial phase of fracture healing, inflammatory cells such as leukocytes, macrophages, and mast cells migrate to the fracture site[2]. Activation of polymorphonuclear leukocytes results in the production of superoxide radicals and initiates the inflammatory response through oxidative stress[5]. In addition, the transient ischemic period following fracture injury, arterial vasoconstriction, and the activation of local hypoxia mechanisms create an environment that increases free radical production[6,7]. Studies have revealed that free radicals negatively affect the bone fracture healing process[8]. Lipid peroxidation has been reported to increase bone resorption by activating osteoclasts[9]. An oxidative stress environment has been reported to disrupt osteoblast and osteoclast functions, leading to decreased bone mass and delayed fracture healing[10]. Clinical studies on older men and osteoporotic individuals have shown that oxidant/antioxidant balance disorder and reactive oxygen species (ROS) formation processes may effectively develop bone loss[11-13].

Silymarin is a natural flavonoid compound with potent antioxidant and anti-inflammatory properties derived from the extract of the *Silybum marianum* (Milk thistle) plant[14]. It is widely used in phytotherapy and traditional medicine practices in the treatment of conditions that adversely affect liver health, such as chronic inflammatory diseases, hepatitis, cirrhosis, and fibrosis[15]. In addition to its hepatoprotective effects, it has also been reported to reduce inflammation, suppress oxidative stress, support bone mineralization, and protect bone density[15-17]. Studies have revealed that silymarin may contribute to the prevention of osteoporosis progression and treatment processes by regulating bone metabolism and reducing oxidative stress levels[16-18]. These studies have focused on the osteogenic properties of silymarin and its antioxidant effects in osteoporosis. In our study, silymarin's ROS-scavenging antioxidant effects on fracture healing

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were evaluated within the context of enzyme levels and radiologic and histopathologic findings.

Materials and Methods

Animals

Ethical approval for the use of experimental animals in this study was approved by Kahramanmaraş Sütçü İmam University Faculty of Medicine Dean's Office Animal Experiments Local Ethics Committee with session number 2015/04. Forty-eight male Wistar-Albino rats with an average weight of 300 g were included in the study. The experimental groups were randomly divided into three groups, with 16 animals in each cage. 48 rats were housed in a controlled environment with a constant temperature of 22 ± 1 °C, humidity of 60 ± 5%, and a 12-h light/dark cycle.

Experimental design

The experimental animals were divided into three groups: Group 1 (Control): In this group, a fracture was induced in the right tibia of 16 randomly selected rats. The animals were given unlimited tap water and standard rodent feed. Group 2 (Sham): A fracture was induced in the right tibia of 16 randomly selected rats, and saline was administered to these animals by gavage. Group 3 (Silymarin): Silymarin (Merck S0292) at a dose of 50 mg/kg/day was administered by gavage to 16 rats with fractures in the right tibia. The 16 rats in each group were divided into two subgroups of eight rats on day 0 and day 21. At the end of the treatment period, the experimental animals were sacrificed under general anesthesia using ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) at a dose of 50 mg/kg intraperitoneally. Biochemical, histopathological, and radiological analyses were performed after the sacrifice.

Biochemical analyses

Tissue samples obtained from the subjects for MDA, SOD, and CAT analysis were homogenized in 1.15% M KCl at 16,000 rpm for 3 min. Homogenates were centrifuged at 14,000 × rpm at +4˚C, and all subsequent analyses were performed with the supernatant obtained. Protein analysis of the samples was conducted using the widely used Folin-Ciocalteu method[19]. The principle of this method is based on the fact that the complex formed with copper sulfate solution added to the protein solution reacts with the Folin-Ciocalteu reagent to produce a blue colour. The absorbance value of the blue colour formed in the samples was measured spectrophotometrically at 750 nm wavelength, and protein concentration was calculated using the standard curve. Malondialdehyde (MDA) levels were determined to assess lipid peroxidation. In the measurement of MDA level, the reaction with thiobarbituric acid (TBA) at low pH (3.4) is taken as a basis. As a result of this reaction, the pink-coloured complex formed between MDA and TBA was analyzed spectrophotometrically at a wavelength of 532 nm[20]. In the measurement of superoxide dismutase (SOD) activity, xanthine and xanthine oxidase systems were used to generate superoxide radicals. The colour intensity of the blue-coloured formazan complex formed by the superoxide radicals reacting with nitroblue tetrazolium (NBT) was measured at 560 nm wavelength[21] . 5.6 U/mL SOD activity of the samples was quantitatively determined using the curve generated with the Ransod kit standard. The catalase (CAT) enzyme activity is measured based on the reaction of hydrogen peroxide to water and oxygen[20,21]. The kinetics of this reaction was analyzed by spectrophotometric measurement of the decrease in the amount of hydrogen peroxide at a wavelength of 240 nm.

Histological analysis and assessment

Bone tissue samples from the fracture site were fixed in 10% neutral buffered formaldehyde solution for 24-h for histologic examination. All specimens were routinely monitored using the tissue tracking device, and paraffin blocks were prepared. From these paraffin blocks, serial sections of 4-6 μm thickness were prepared for each tissue sample with a microtome and stained with Hematoxylin-Eosin (H&E) stain. The preparations were subjected to histopathologic examination using light microscopy. The groups were evaluated regarding vascularization, fibrocartijelanosis, osseous callus, and osteoblastic activity.

Radiological assessment

Rats in the control, sham, and silymarin groups were sacrificed on day 0 and day 21 of the treatment. After the procedure, the soft tissues surrounding the right posterior tibias were carefully cleaned. The tibias were placed in X-ray cassettes, and direct radiographs were taken from anteroposterior and lateral planes. A specialized radiologist evaluated all radiographic images.

Statistical analysis

Shapiro-Wilk tests were used to assess the normality of the data. One-way ANOVA analysis of

variance was used for group statistical comparisons, followed by Dunnett's multiple comparison tests. If the data did not follow a normal distribution, the Kruskal-Wallis and Dunn's post-hoc tests were used. The data were expressed as mean and standard deviation. A *P*-value less than 0.05 was considered significant and denoted as **P*<0.05, ***P*<0.01, or ****P*<0.001.

Results

Biochemical analyses

Antioxidant and oxidant parameters were analyzed on the zero and twenty-first days after fracture to evaluate the tissue redox status in rats with the tibial fracture model. According to the day zero results, a significant increase was found in SOD enzyme activity in the silymarin group compared to the control group (*P*<0.001) (Fig. 1A). However, no significant difference was observed between the control and sham groups in terms of SOD activity (ns). When the twenty-first day SOD activities were compared, a substantial increase was determined in silymarin and sham groups compared to the control group (*P*<0.001) (Fig. 1D).

When the groups' CAT activities were evaluated, an increase in CAT activity was observed in both sham and silymarin groups compared to the control group on day zero. However, this increase was not statistically significant (ns). On the twenty-first day, CAT enzyme activity was found to be significantly higher in sham and silymarin groups compared to the control (*P*<0.001) (Fig. 1B & 1E). Day zero results

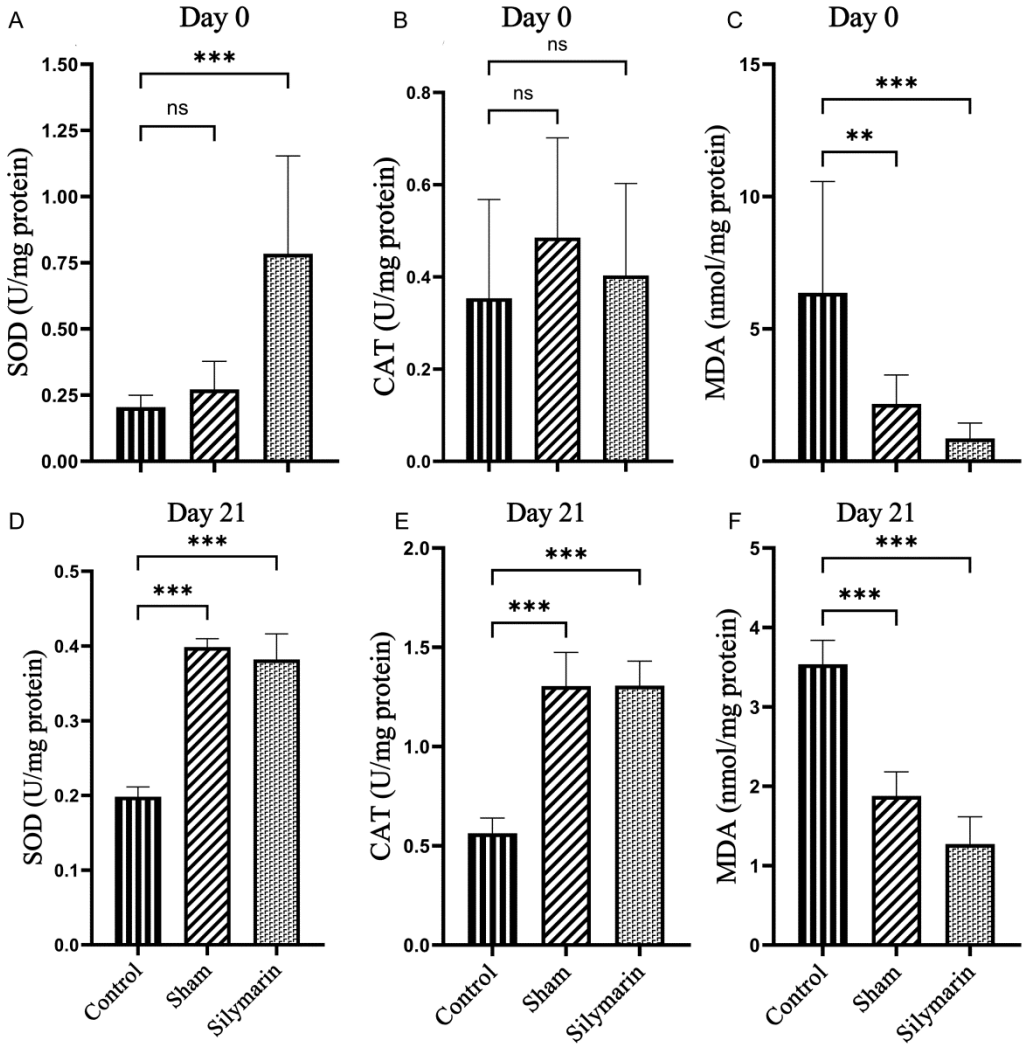

Fig. 1 — Comparison of SOD, CAT, and MDA levels of control, sham, and silymarin groups on day zero and twenty-one after tibial fracture. Following silymarin administration, antioxidant enzyme levels increased, and lipid peroxidation decreased compared to the control group. [ns: not significant, **P*<0.05, ***P*<0.01, or ****P*<0.001]

showed that oxidative stress levels were high immediately after fracture formation, and antioxidant defense mechanisms were partially activated (Fig. 1A & 1B). Twenty-first-day results showed that silymarin treatment significantly reduced oxidative stress levels during the healing process (Fig. 1D & 1E).

When MDA levels, which is an important indicator of lipid peroxidation, were evaluated, it was found that MDA levels in the silymarin group were significantly lower compared to the control group on both day zero and day twenty-first ($P<0.001$) (Fig. 1C & 1F). On day zero and day twenty-one, MDA levels in the control group were significantly higher than in the silymarin and sham groups (**$P<0.01$, or ***$P<0.001$) (Fig. 1C & 1F). The findings indicate that lipid peroxidation significantly increases after tibial fracture and silymarin treatment decreases lipid peroxidation in the early twenty-one days of healing. The results in Table 1 show the high MDA level in the control group on days zero and twenty-one and the increased oxidative stress after tibial fracture. Silymarin supported the oxidative defense system by increasing SOD and CAT activities partially on day zero and significantly on day twenty-one and succeeded in keeping MDA levels low in general.

Histological analysis and assessment

Histopathological evaluations were performed based on the parameters of vascularisation, fibrocartilagous tissue, osseous callus formation, and osteoblastic activity in bone tissue. Analysis of fracture line healing at the end of the twenty-first day revealed minimal cartilage development and slightly increased fibroblastic activity and vascularisation in the Sham group compared to the control group (Fig. 2). These findings indicate that the bone healing process in the Sham group was in the initial stages and did not reach a complete osseous formation. In the silymarin group, on the other hand, callus formation progressed significantly; the callus area was larger, and the density was higher. This suggests that silymarin may play a role as an effective biomodulator supporting osteogenesis processes. In addition, it was noted that osteoblastic activity increased significantly in the silymarin group, and osseous tissue was formed within 21 days. These results suggest that silymarin may accelerate osseous tissue formation by promoting osteoblast proliferation and differentiation. Compared to the sham group, it was found that fibroblastic activity was higher in the Silymarin group, resulting in the formation of

Table 1 — Comparison of SOD, CAT and MDA levels of control, sham and silymarin groups on day 0 and 21 after tibial fracture

	Groups	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nmol/mg protein)
Day 0	Control	0.20 ± 0.045	0.35 ± 0.21	6.4 ± 4.2
	Sham	0.27 ± 0.11	0.49 ± 0.22	2.2 ± 1.1
	Silymarin	0.78 ± 0.37	0.40 ± 0.20	0.86 ± 0.58
Day 21	Control	0.20 ± 0.013	0.56 ± 0.076	3.5 ± 0.30
	Sham	0.40 ± 0.011	1.3 ± 0.17	1.9 ± 0.30
	Silymarin	0.38 ± 0.034	1.3 ± 0.12	1.3 ± 0.35

[Data are presented as mean ± standard deviation (SD) (n=8). In the control group, high MDA levels on days zero and twenty-one were indicative of oxidative stress. Silymarin supported the oxidative defence system by increasing SOD and CAT activities partially on day 0 and significantly on day 21]

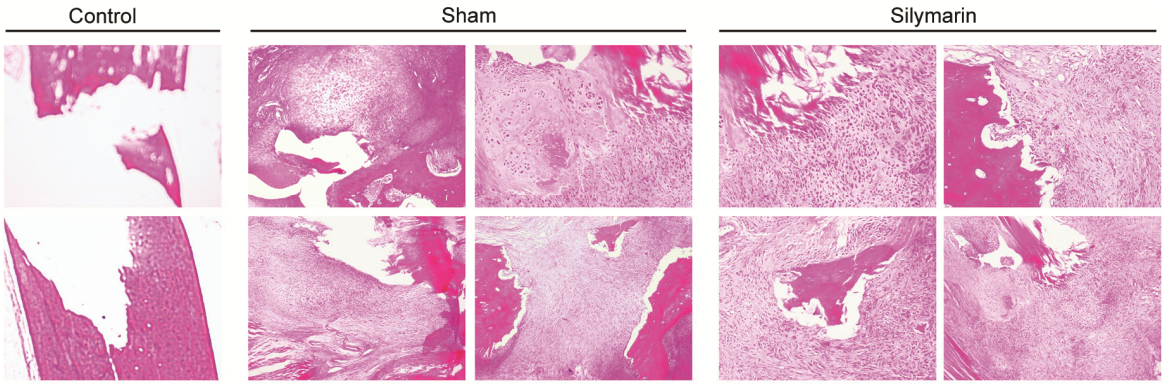

Fig. 2 — Comparison of histopathological changes in control, sham, and silymarin groups after fracture formation. On day 21, minimal cartilage development and a limited increase in fibroblastic activity were observed in the Sham group, while a significant increase in fibroblastic activity, advanced cartilage formation, osseous development, and new bone formation were detected in silymarin group.

prominent cartilage tissue, osseous development, and organized bone tissue formation. The findings indicate that silymarin treatment increases callus formation, contributes to bone tissue remodeling, and accelerates bone regeneration.

Radiological assessment

On the radiographic examinations performed on day zero, a displaced single-line fracture was detected in the diaphyseal region of the tibiae of the control, sham, and silymarin groups. These observations confirm that the experimental model was successfully established. On the twenty-first day of radiographs of the control group, it was determined that the fracture lines of both tibias continued clearly, and the healing process was limited (Fig. 3). In the radiographs of the sham group, it was observed that the visibility of the fracture lines decreased, but callus formation was partial, and bone integrity was not completely achieved. In the radiographic examinations performed in the silymarin group, it was determined that the fracture lines completely disappeared, the callus density increased, and the bone tissue reconstruction was advanced. These findings indicate that silymarin application accelerated fracture healing, and morphological healing was more pronounced.

Discussion

Although fracture injuries vary according to age and clinical case characteristics, they are among the

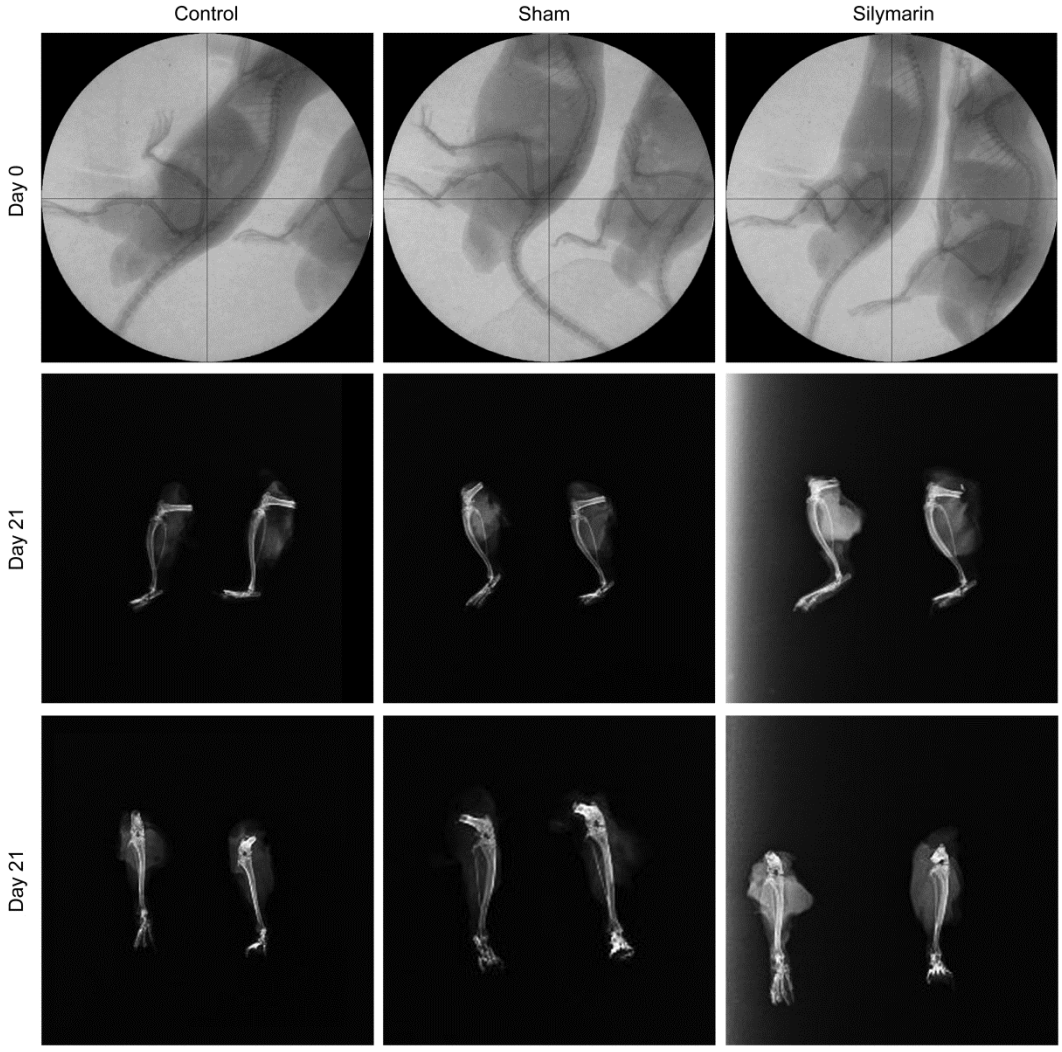

Fig. 3 — Radiographic examinations of the control, sham, and silymarin groups were performed on the zero and twenty-first day after the tibial fracture. On the radiographic examinations performed on day zero, a displaced single-line fracture was detected in the diaphyseal region of the tibiae of the control, Sham, and Silymarin groups. At the end of twenty-one days in the silymarin group, it was observed that the fracture lines disappeared, callus density increased, and bone tissue was reorganized.

most common orthopedic pathologies[22]. Clavicle fractures in the neonatal period, long bone fractures due to falls in childhood, femur and distal radius fractures due to trauma in adults, and hip and spine fractures due to osteoporosis in elderly individuals are among the most common types of fractures[23]. To simulate fracture injury, a closed fracture was created in the right tibia of rats in our study using a bending method with a system designed according to the special metal three-point principle. This method allowed the modeling of long bone fractures by creating a controlled tibia fracture with minimal trauma to the surrounding tissues. Systemic complications such as vascular injury, ischemia, nerve tissue damage, and loss of bone density mainly occur after tibial fractures[24]. This leads to increased inflammatory response in tissues, accelerated neutrophil infiltration, and increased free oxygen radical production at the cellular level[25]. In this context, the protective potential of silymarin against the negative effects of fracture injury on bone and fracture metabolism was evaluated through antioxidant/oxidant balance. Silymarin, frequently preferred in managing inflammatory liver diseases in traditional medicine, was evaluated for its antioxidant and potential anti-osteoporotic effect in this study. Preclinical, experimental studies have revealed the protective effect of silymarin against various pathologies such as liver and kidney diseases[26], inflammatory bowel diseases[27], diabetes complications[28], peptic ulcer[29], and atherosclerosis[30] through antioxidant and anti-inflammatory molecular mechanisms. However, the potential role of the antioxidant properties of silymarin in the clinical management of liver diseases has also been studied in detail in placebo-controlled phase studies[14,31]. Although it is reported in the literature that the antioxidant properties of silymarin are effective in preventing and treating various diseases, studies examining its effect on fracture injuries are quite limited. In a study in osteoblastic mouse cells and *in vivo* fracture models, silymarin supplementation was reported to increase alkaline phosphatase and osteocalcin levels, accelerate osteoblast proliferation, and promote bone regeneration by activating BMP/SMAD/Runx2 signaling pathways[18]. Silymarin has been reported to be associated with increased BMP-2 and Runx2-targeted osteoblast activation and alkaline phosphatase levels. This cascade effect has been reported to enhance fracture healing in mouse tibias by promoting mineralization, Type I collagen release, and calcium deposition in the extracellular matrix[18]. Silibinin administered at a dose of 150 mg/kg/day to rats with diabetic periodontitis supported mitochondrial biogenesis by increasing the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), thereby promoting osteogenesis and preventing bone loss[32]. In a study conducted in mice with postmenopausal osteopenia model, it was reported that milk thistle extract could prevent estrogen deficiency-induced bone loss by reducing the nuclear factor-κB ligand/osteoprotegerin ratio and suppressing osteoclastogenic activity modulators such as TRAP and cathepsin K[33]. In same study, it was reported that the application of milk thistle containing silibinin reduced osteoclastogenesis by decreasing the serum RANKL/OPG ratio in ovariectomized mice and that this application may prevent the development of postmenopausal osteoporosis caused by estrogen deficiency[33]. In addition, bone-forming and osteoprotective effects of silibinin, one of the active components of silymarin, on murine osteoblastic cells have been investigated. Studies have reported that silybin increases calcium accumulation, promotes bone nodule formation and matrix mineralization, and may offer a potential therapeutic approach by supporting osteoblastogenesis[34]. These studies primarily focused on osteoporosis and bone integrity abnormalities that lead to bone fragility. In our study, MDA, SOD, and CAT levels, histological and radiological examinations were evaluated together in tibial fractures, and the effects of silymarin on bone regeneration were assessed in detail through oxidative stress parameters and bone tissue healing. Our study found that silymarin stimulated bone formation and supported osteoprotective effects by increasing levels of oxidant suppressor enzymes from day zero to day twenty-one. SOD, CAT, and MDA activities on day zero indicate that antioxidant defense mechanisms are not yet fully activated following fracture formation. However, a significant increase in antioxidant enzyme levels and a significant decrease in lipid peroxidation were determined in the silymarin-treated group on day 21. Histological and radiological examinations performed on the twenty-first day revealed that the increase in SOD and CAT antioxidant enzyme levels in the silymarin group occurred in parallel with fracture healing. The fact that MDA is one of the important indicators of oxidative stress has been

supported by many studies in the literature. On the twenty-first day, the significantly lower MDA level in the silymarin group was associated with decreased oxidative stress. This was also consistent with the histopathological increase in fibroblastic activity and development of cartilage tissue. Our study revealed that silymarin administration positively affected bone metabolism by decreasing oxidative stress and promoting tissue healing.

In the literature, it has been reported that the formation of free oxygen radicals continues for one month following the fracture, and this process covers both inflammation and repair periods[35,36]. In studies conducted in rats with an experimental fracture model, it was shown that erythrocyte MDA levels increased on the 5th, 10th, 20th, and 30th days after fracture, and oxidative stress continued throughout the healing process; however, the endogenous antioxidant system was insufficient to balance this situation[35]. In another study, it was reported that malondialdehyde (MDA) levels increased on days 1, 3, 7, 14, and 28 in bone tissue of rats with tibial fracture, reflecting the increase in oxidative stress levels due to fracture formation[36]. It has been reported that endogenous antioxidant defense mechanisms may be insufficient to control oxidative stress, which increases in the first month of fracture healing. Therefore, various antioxidant supplements may be beneficial[35,37]. In this context, the effects of agents with free radical scavenging properties such as α-Tocopherol, vitamin C, ginsenoside, and edaravone on reducing oxidative stress and supporting bone repair during the fracture healing process have been investigated[38-41]. Vitamin C treatment administered to rats with tibial fractures for 21 days was reported to accelerate new bone formation and support callus development[40]. In another study, the healing process of osteoporotic fractures of α-Tocopherol was evaluated after 14 days of administration. It was reported that α-Tocopherol supplementation increased antioxidant enzyme activities in osteoporotic bones by reducing excessive free radicals formed in the early period of fracture healing[39]. In addition, histological evaluations have demonstrated that edaravone, which can neutralize free radicals, has positive effects on the fracture healing process after tibia fracture[41]. The study conducted by our team supports previous studies, which show that oxidative stress increases after fracture formation and that using agents that support antioxidant defense mechanisms in the first month of fracture healing positively affects callus formation. However, the novelty of our current study is that, unlike the antioxidant agents that have been predominantly studied in the literature, it strengthens antioxidant defense at the molecular level and supports fracture healing in histological and radiological parameters. SOD, CAT, and MDA levels measured in bone samples taken from fractured tibiae revealed that silymarin contributed to the activation of antioxidant mechanisms in the early healing period, while our histopathological and radiological findings showed that silymarin increased callus density, accelerated osseous development and supported the formation of organized bone tissue, revealing holistic antioxidant response mechanism to the existing literature.

Although milk thistle and silymarin are frequently used as hepatoprotective agents, pharmacokinetic data obtained in vivo and human subjects do not provide a common systematic summary[42]. Silybum marianum flavonolignans have good oral bioavailability and gastrointestinal absorption and are conjugated and metabolized in the liver, particularly by phase II reactions[43]. Despite in vitro data showing interactions of silymarin or individual flavonolignans with various enzymes and transporters, silymarin-drug interactions in human studies are not significant from a clinical perspective[42]. While pharmacokinetic data observed in vivo, in vitro, and human studies reveal that silymarin contains uncertainties in terms of its systemic interactions, data based on molecular interactions indicate that the antioxidant effects of silymarin contribute to the reduction of oxidative stress[44].

Conclusion

This study reveals that silymarin administration positive affects on bone metabolism by reducing oxidative stress and promoting tissue healing. With its free radical scavenging properties, silymarin supports bone repair processes in the early period of fracture healing. However, further studies evaluating the cumulative effects of silymarin on the fracture healing process, encompassing both bone tissue and surrounding tissues in ischemia, osteoporosis, and other fracture injury models, will contribute to the development of innovative strategies for fracture treatment. In addition, evaluating different dosage levels and prolonged recovery times with larger sample groups in future studies will enhance the generalisability of the findings obtained and allow the determination of more effective strategies in clinical applications.

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Conflict of interest

The authors declared that they have no conflict of interest.

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