İst Tıp Fak Derg 2018
J Ist Faculty Med 2018

ARAŞTIRMA / RESEARCH
DOI: 10.26650/IUITFD.394531

TAURIN KIRIK İYİLEŞMESİNİ ETKİLER Mİ? DENEYSEL BİR ÇALIŞMA

DOES TAURINE IMPROVE FRACTURE HEALING? AN EXPERIMENTAL STUDY

Elçil KAYA BİÇER*, Nilgun YENER**, Başak DOGANAVSARGIL***, Mehmet ARGIN****, Akın KAPUBAGLI*

Ege University, Faculty of Medicine, *Department of Orthopaedics and Traumatology, **Department of Clinical Pathology and ***Department of Radiology, İzmir, Turkey
****Dokuz Eylul University, Faculty of Medicine, Department of Biochemistry, İzmir, Turkey

Cite this article as: Kaya Biçer E, Yener N, Doğanavşargil B, Argın M, Kapubağlı A. Does taurıne improve fracture healing? an experimental study. J Ist Faculty Med 2018; 81(3): 91-98

ABSTRACT

Objective: To evaluate the influence of taurine, a non-structural amino acid with antioxidant effects, on fracture healing.

Materials and Methods: Open tibial osteotomy was performed on nine New Zealand rabbits in the control group and seven in the taurine group. Radiologic fracture healing was assesed using the Goldberg score on x-rays and computed tomography (CT) sections on the 21st day. Healing was graded histologically using the Huo score. Serum malondialdehyde (MDA) and alkaline phosphatase (ALP) levels were measured, at days zero, seven, 14 and 21.

Results: Upon completion of the study, in the taurine group, the median stage of healing was recorded as "radiologic union", whereas it was "probable union" in the control group with respect to x-ray and CT sections. In the taurine group, callus was mainly composed of osteoid tissue (median Huo score 8); in the control group the predominant tissue was chondroid (median Huo score 6). Changes in MDA levels revealed that oxidative stress was greatest in the inflammatory phase. In both groups, the ALP levels first decreased, and then increased ,with new bone formation on the 21st day.

Conclusion: Taurine improved early bone healing in an experimental animal osteotomy model. This influence might be related to its antioxidant properties.

Keywords: Taurine, fracture healing, oxidative stress, antioxidants, malondialdehyde (MDA)

ÖZET

Amaç: Yapısal olmayan bir amino asit olan antioksidan etkili taurinin kırık iyileşmesi üzerine etkilerinin araştırılması

Gereç ve Yöntem: Kontrol grubunda dokuz, taurin grubunda yedi tane Yeni Zelanda tipi tavşana açık tibial osteotomi uygulandı. Radyolojik kırık iyileşmesi, 21. günde çekilen direk grafi ve bilgisayarlı tomografi (BT) kesitleri üzerinde Goldberg skoru ile değerlendirildi. Histoloik olarak iyileşme Huo skoruna göre derecelendirildi. Sıfırıncı, yedinci, 14. ve 21. günlerde serumda malondialdehit (MDA) ve alkalen fosfataz (ALP) düzeyleri ölçüldü.

Bulgular: Direk grafi ve BT kesitlerine göre kırık iyileşmesinin ortanca evresi taurin grubunda radyolojik kaynamayken kontrol grubunda olası kaynama olarak bulundu. Histolojik olarak, kallus taurin grubunda esas olarak kemik dokusundan oluşurken (ortanca Huo skoru 8), kontrol grubunda baskın doku kıkırdaktı (ortanca Huo skoru 6). MDA düzeyindeki değişimler oksidatif stresin kırık iyileşmesinin enflamatuvar fazında en yüksek olduğunu göstermekteydi. Her iki grupta da ALP düzeyleri önce azalmış, 21. Günde yeni kemik oluşumuyla yeniden yükselmiş olarak bulundu.

Sonuç: Bu çalışmanın sonuçları deneysel osteotomi modelinde taurinin erken evre kırık iyileşmesi üzerinde olumlu etkileri olduğunu desteklemektedir. Bu etki tarunin antioksidan özelliği ile ilişkilendirilebilir.

Anahtar Kelimeler: Taurin, kırık iyileşmesi, oksidatif stres, antioksidan, malondialdehit (MDA)

INTRODUCTION

Bone healing is related to a multitude of conditions, and treatment modalities in medicine. It begins with fracture and hematoma formation, followed by inflammation, the formation of cartilaginous and bony callus, and then remodeling. A variety of cell lines, biomolecules, their receptors, as well as extracellular medium and their interactions concert together (9).

İletişim kurulacak yazar/Corresponding author: elcil@yahoo.com
Geliş tarihi/Received Date: 13.02.2018 • **Kabul tarihi/Accepted Date:** 15.05.2018

The exact mechanism by which bone healing occurs is not fully understood. 5 to 10 percent of fractures result in delayed healing or nonunion despite proper treatment (25). Several local and systemic factors are known to affect bone healing. Patient related factors are added to by extrinsic factors such as severity and degree of injury to the bone and surrounding soft tissues, as well as reduction and fixation of fracture. Current literature points at free oxygen radicals (the by-products of many biochemical reactions occurring throughout the injury and healing process), as being another factor (8, 11).

Involvement of free oxygen radicals is not necessarily harmful; the proinflammatory cells and osteoclasts depend exclusively on these molecules - especially in the inflammatory phase of bone healing (5, 10, 29, 30). Oxidative stress (which is defined as disruption of the fine equilibrium between free radical formation and neutralization of their excess at molecular level), however, is found to delay bone healing (8, 11).

Vitamin C, vitamin E, allopurinol and N-acetyl cysteine are among the antioxidant molecules tested for positive contributions to bone healing. Although there is a consensus on the effects of vitamin C, the other molecules yielded contradictory results (7, 13, 18, 26, 28, 32, 33, 36).

The aim of this study is to investigate the effects of taurine, a non-structural semi-essential amino acid with known antioxidant properties, on the bone healing process.

MATERIALS AND METHODS

The study commenced with the approval of the local ethical committee approval. There were nine New Zealand type adult male rabbits in the control group and seven in the taurine group. Along with daily sewage and ambient illumination respecting their diurnal rhythm, drinking water was supplied ad libitum. Barley, cereal oats, and dried shamrock were supplied as fodder.

A standardized right tibial osteotomy was performed on all of the subjects. Osteosynthesis was achieved by an intramedullary Kirschner wire (Figure 1 a-ı). The control group (Group 1) received 5 ml tap water via orogastric tube for five days in the postoperative period. The study group (Group 2) received taurine (18 g/100 mL of water, 2-aminoethansulphonic acid, Applichem, Darmstadt, Germany). The drug administration method and timings were the same as the control group. The daily dose adjustment was set to 300 mg/kg/day.

Two milliliters of blood were collected from the ear veins of all the subjects before surgery for baseline values. Blood sampling was repeated at days seven, 14, and 21 postoperatively.

At postoperative day 21, the subjects were euthanized by barbiturate injection. Both cruri were disarticulated at the knee and ankle joint post mortem. Soft tissue was carefully removed so as not to damage any callus formation present on the tibia. The Kirschner wires were removed. The right tibiae were subject to direct X-ray imaging and computed tomography scans. Following radiological assessment, the tissues were isolated for histological examination.

Radiological Assessment

Direct X ray images were obtained at day zero and 21 postoperatively. Day zero images were obtained to assess the status of the fracture and fixation (Figure 2a, b). Day 21 images were obtained post mortem utilizing a Kodak® Directview CR975 ™ system (Carestream Health,

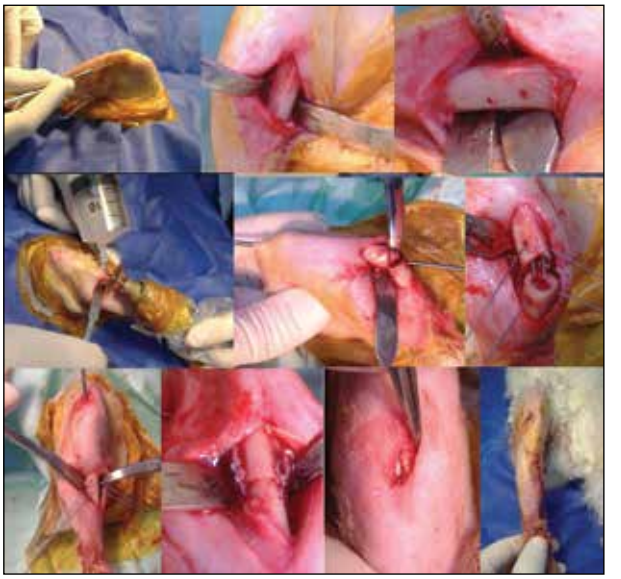

Figure 1. a-ı. Surgical technique of tibial osteotomy and osteosynthesis. Skin incision (a), proximal tibia was exposed (b), two holes were drilled on both anteromedial and lateral sides of tibia to maintain rotation (c), osteotomy of tibia (d), intramedullary propagation of K wire into the proximal segment (e), passage of sutures through the holes on the tibia (f), intramedullary fixation of the osteotomy with the K wire (g), the sutures were tied in order to control rotation at the level of osteotomy (h), after closure of the skin incision (ı).

Figure 2. a, b. Postoperative control x-rays. Anteroposterior (AP) view (a), lateral view (b).

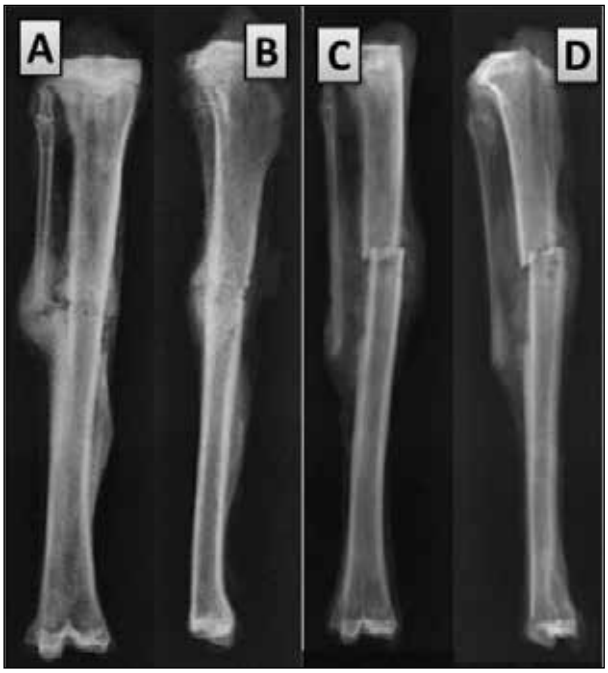

Figure 3. a-d. Postoperative 21st day x-rays. AP view of a grade 3 union (taurine group) (a), lateral view of a grade 3 union (taurine group) (b), AP view of a grade 2 union (control group) (c), lateral view of a grade 2 union (control group) (d).

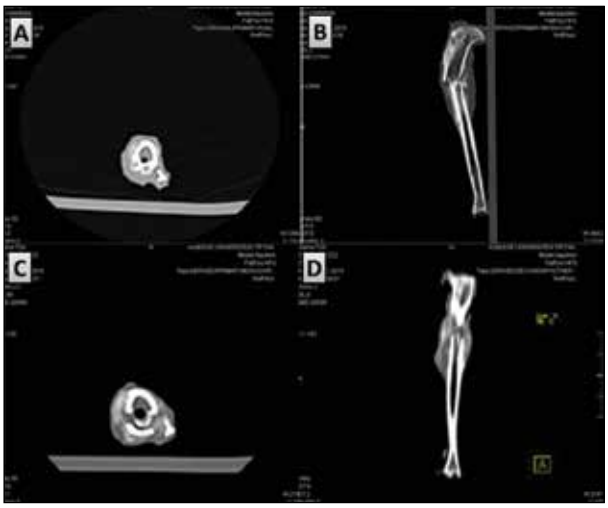

Figure 4. a-d. CT scan images, axial and sagittal views. Axial image, grade 2 union (a), sagittal image, grade 2 union (b), axial image, grade 3 union (c), sagittal image, grade 3 union (d).

Inc., Rochester, New York, USA). The degree of bone healing was graded according to the Goldberg classification (12). Scoring was made by two blinded observers (a radiologist specialized in musculoskeletal radiology and an orthopaedic surgeon).

Computerized tomography (CT) sections were obtained post mortem with a Toshiba ® Aquilion 16™ detector equipped device (Toshiba Medical Systems Corporation, Tochiyiken, Japan) under standard conditions (120 kV, 100 mA, 0.5 msec, 50 mAs). Images were obtained with 0.5 mm slice thickness and 0.1 mm intervals. Images were processed with Siemens ® Syngo Workstation ™ program (Siemens Medical Solutions, Erlangen, Germany). The mineral density of the callus tissue was measured in Hounsfield Units (2). The axial sections were selected where calcification was more obvious to visual observation and the average densities of three random points were calculated. The density of the callus tissue over the cortical bone was calculated.

Histological Assessment

Both tibiae of the subjects were assessed histologically. Following fixation in a 10% buffered neutral formaldehyde solution, the decalcification process was continued for seven days in a 20% formic acid solution. The specimens were embedded in a paraffin block. Deparaffinization was accomplished by obtaining 4 µm thick slices and staining by Hematoxylin & Eosin (H&E).

A standard light microscope was used to examine the slices (Olympus BX50, Olympus Corporation, Tokyo, Japan). Histometric analyses were carried out using images obtained by a camera (Carl Zeiss Axiocam HRC3, Carl Zeiss Microimaging GmbH, Göttingen, Germany) and a microscope (Zeiss AX10, Carl Zeiss Microimaging GmbH, Göttingen, Germany) under 4x magnification. The images were evaluated by Axiovision rel. 4.8 ™ software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). The phases of bone healing were investigated in these images utilizing the Huo score (14). The total area of fibrous, cartilaginous, and osseous tissue - aside from the cortical bone and marrow - was regarded as callus tissue and labeled as such on digital images. Individual areas of each of these tissue types were measured in proportion to total callus area and documented in percentages.

Biochemical Analyses

Following five minutes of centrifugation at a speed of 4000 rpm (Heraeus Biofuge Stratos Centrifuge, Kendro Laboratory Equipments, Sollentum, Germany), serum alkaline phosphatase (ALP) levels were studied on the same day the samples were collected in order to avoid the risk of enzyme denaturation. The remaining serum was isolated for preservation in -80 °C (Forma ®Powerfreeze™ -86 °C ULT Freezer, Thermo Electron Corporation, Waltham, Massachusetts, USA) for lipid peroxidation tests, which were performed at the end of the study.

Serum ALP levels were measured with a para nitrophenyl phosphate reaction in a clinical chemistry analysis device

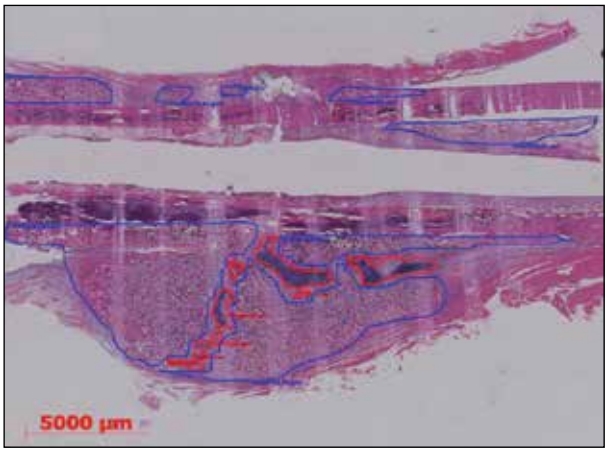

Figure 5. Histologic assessment of stages of fracture healing; marking and calculating the areas of cartilage (red lines) and osteoid tissue (blue lines).

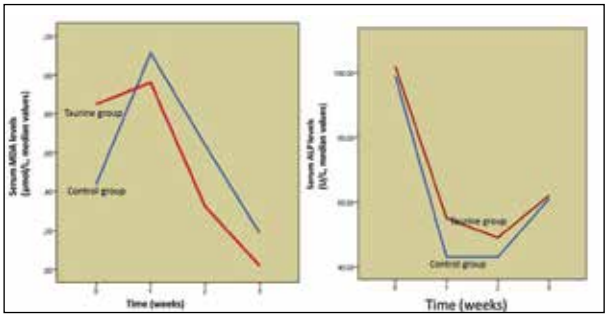

Figure 6. a, b. Serum MDA levels throughout the study period (a), Serum ALP levels throughout the study period (b).

(Architect c16000 Clinical Chemistry Analysis™ Device, Abbott Laboratories, Abbott Park, Illinois, USA).

Lipid peroxidation was assessed indirectly by measuring serum malondialdehyde (MDA) levels. These measurements were carried out using a high-performance liquid chromatography (HPLC) device (17).

Statistical Analysis

Obtained data was analyzed by Statistical Packages for the Social Sciences program version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at 0.05 and the distribution of data was tested with the Kolmogorov Smirnov test.

Goldberg scores and bone mineral densities were tested with the Mann Whitney U test, while the correlation between X-ray and tomography findings was investigated using Spearman's correlation analysis. Inter-observer agreement was tested by Cohen's kappa coefficient.

Histological findings such as the amount of fibrous tissue, cartilaginous tissue, osteoid tissue ratios, and level of bone healing graded according to Huo scores were compared using the Mann Whitney U test.

Serial values obtained for MDA and ALP were controlled with the Friedman test for in-group consistency. In-group bilateral comparisons were made with the Wilcoxon signed-rank test. Intergroup comparisons were made with the Mann Whitney U test.

RESULTS

Radiological Findings

In the 21st day x-rays, the median grade of union was 2 (min 2, max 3) in the control group which corresponded to probable union. Median grade was 3 (min 2, max 3) in the taurine group which was determined as radiological union (Figure 3 a-d). The union obtained in the taurine group was found to be significantly more pronounced by both of the observers (1st observer p=0.015, 2nd observer p=0.042). Kappa statistics revealed that there was a high level of agreement between the two observers (kappa= 0.625, p=0.012). Remodeling was not observed in any of the subjects.

In line with the x-rays, union was more pronounced in the taurine group with respect to the evaluation of CT images (Figure 4). In the taurine group the median grade of union was 3 (min 3, max 3); however, it was 2 (min 2, max 3) in the control group (p=0.008). The classification of union on x-rays and CT scans were highly correlated with each other (rs=0.775, p=0.010).

In the taurine group, both the mineral density of the callus tissue and its ratio was found to be higher despite this not being statistically significant (median density, group 1: 719.33 HU (min 498.00, max 1200.67), group 2: 842.00 HU (min 498.67, max 1065.33), p=0.427; density ratio (median), group 1: 26% (min 20%, max 45%), group 2:29% (min 19%, max 42%), p=0.266).

Histological Findings

The median Huo score of the control group was 6 (min 3, max 8); whereas it was 8 (min 6, max 8) for the taurine group. The histological degree of bone healing was significantly better for the taurine group when compared to the control (p=0.021). These findings matched the calculated tissue type ratios as well. At the 21st day, the callus was predominantly composed of osteoid tissue with small amounts of cartilage in the taurine group. However, in the control group the predominant tissue type of the callus was cartilage with small accompanying fibrous and osteoid tissue areas. The tissue type ratios are given in table 1 (Figure 5). The comparison of tissue type ratios revealed that the osteoid tissue ratio of the callus was significantly greater in the taurine group (p=0.013). Fibrous and cartilaginous tissue ratios were not signifi-

Table 1. Tissue type ratios

		Control group (n=9)	Taurine group (n=7)	p
Fibrous tissue ratio	Mean±SD	18.97%±28.29%	0%±0%	
	Median	0%	0%	0.052
	Min-max	0%-66.12%	0%-0%	
Cartilage tissue ratio	Mean±SD	60.84%±28.15%	32.67%±32.35%	
	Median	67.62%	12.20%	0.064
	Min-max	20.25% - 93.83%	8.94%-88.05%	
Osteoid tissue ratio	Mean±SD	20.19%±25.60%	67.34%±32.35%	
	Median	12.56%	87.80%	0.013*
	Min-max	0%-79.75%	11.95%-91.06%	

SD: Standard deviation

Table 2. Serum MDA levels (µmol/L)

MDA levels		Control group (n=9)	Taurine group (n=7)	p
Day 0	Median	1.438	1.850	0.958
	Min-max	1.227-2.480	0.659-2.012	
Day 7	Median	2.112	1.961	0.791
	Min-max	1.185-13.998	0.992-16.182	
Day 14	Median	1.640	1.324	0.223
	Min-max	0.985-4.211	0.696-1.785	
Day 21	Median	1.187	1.019	
	Min-max	0.615-2.576	0.481-2.118	0.560
	p	0.012*	0.008**	

*Statistically significant difference
Post hoc analyses: Day 7 vs day 21 p=0.008
** Statistically significant difference
Post hoc analyses: Day 7 vs days 14& 21 p values 0.018
MDA: Malondialdehyde

cantly different between the groups (p=0.052, and 0.064, respectively).

The histological evaluation of the left intact tibiae of subjects revealed that disturbances of bone turnover which might have influenced the stages of fracture healing were not present in either group.

Biochemical Findings

MDA levels are summarized in table 2. The changes in serum MDA levels were found to be statistically significant for both groups (group 1, p= 0.012; group 2, p= 0.008). In-group analyses revealed that for the control group, the difference between days 7 and 21 was significant (p=0.008). In the taurine group both days 14 and 21 the MDA levels were significantly lower than day 7 values (p=0.018 for both). Intergroup analyses did not reveal any significant difference statistically (p>0.05). Nevertheless, when compared with day 0, at the 7th day the increase in the MDA levels was lower in the taurine group than the control group. Furthermore, at the 14th and 21st days MDA levels dropped to lower levels in the taurine group (Figure 6a).

Table 3. Serum ALP levels (U/L)

ALP levels		Control group (n=9)	Taurine group (n=7)	p
Day 0	Median	99	102	0.560
	Min-max	39-192	52-169	
Day 7	Median	43	55	0.596
	Min-max	34-126	24-76	
Day 14	Median	43	49	0.596
	Min-max	27-95	21-110	
Day 21	Median	61	62	
	Min-max	33-108	38-121	0.672
	p	0.016*	0.001**	

*Statistically significant difference
Post hoc analyses: Day 0 vs day 14 p=0.008
Day 14 vs day 21 p=0.028
** Statistically significant difference
Post hoc analyses: Day 0 vs days 7&14, day 14 vs day 21 p=0.018
ALP: Alkaline phosphatase

ALP levels are summarized in table 3. In-group analyses revealed a significant change throughout the study for both groups (group 1, p=0.016, group 2, p=0.001). Serum ALP levels were found to decrease markedly from day 0 to day 7, then decrease gently until day 14, with a marked increase afterwards (Figure 6b). However, the difference between the groups was insignificant.

DISCUSSION

The effects of oral administration of taurine on early stages of bone healing were investigated in this experimental fracture model using rabbits as subjects. When the study ceased on the 21st day, a callus including a fully-fledged lamellar bone was not observed. Importantly, however, a measurable callus comprising a mixture of fibrous, cartilaginous, and osteoid tissue components in varying degrees was observed. Histological and radiological findings in this study showed that taurine was beneficial in the early phases of bone healing.

Biological insufficiencies may play a detrimental role in bone healing. A healthy fracture healing is a process where many local and systemic factors interact and the early phases of bone healing are critical (4). Biological insufficiencies and their interactions are not fully understood. Ischemia, or reperfusion mechanism, is one such insufficiency. Following a fracture, a reperfusion state occurs where free oxygen radicals are increased in the tissues (23). When the counterbalancing effects of endogenous antioxidant systems are overcome by the production of free radicals, the tissues are subject to oxidative stress (10). The increment of oxidative stress has been shown to impair bone healing (11). Oxidative stress commonly results in tissue destruction through lipid peroxidation. The amount of lipid peroxidation can be measured through analyzing MDA levels. Previous studies revealed an increase in MDA levels post fracture at days 7 and 14, followed by a decrease starting from the 28th day (22, 31, 35). These findings can be interpreted to mean that oxidative stress appears with reperfusion and not with ischemia.

Although taurine plays no part in protein synthesis, it has many other biological functions, e.g. modulation of osmotic homeostasis and calcium homeostasis, membrane stabilization through deflecting lipid peroxidation, and immunomodulation (3, 15, 16, 24, 27, 34). Several cell culture studies concluded that taurine has a role in normal bone metabolism by inducing osteoblasts and inhibiting osteoclasts (15, 20, 21, 24, 37-39).

Histological assessment of callus tissue on the 21st day revealed that taurine-administered subjects produced a callus rich in osseous components, while the control group produced a callus rich in cartilage and fibrous components. The open tibial osteotomy fracture was described thoroughly by Ashurst in 1986 (1). When the histological findings of our study are compared to the timeline presented in this study, the tissue characteristics were consistent with the third week of bone healing under the mechanically unstable conditions for the tau-

rine group, while the control group exhibited second week characteristics. Moreover, radiological assessments made with x ray imaging and CT scans revealed that the degree of bone healing was better in the taurine group.

Antioxidant effects of taurine were tested with serum MDA levels in this study. The changes in serum MDA levels in serial measurements revealed that oxidative stress was greatest in the inflammatory phase of the fracture healing. Oxidative stress was also present during the repair phase but not as high as in the inflammatory phase. Even though a statistically significant difference could not be demonstrated between the groups, the median levels of the taurine group were measured lower than that of the control group at all points aside from day zero.

In bones, ALP is principally responsible for the mineralization of the skeleton and is associated with bone formation. Besides growth spurt periods, ALP which is synthesized by the osteoblasts reaches higher serum levels when active bone formation occurs in the body (6). Even though not as specific as bone specific ALP, total ALP levels have proven themselves as indicators of bone formation during fracture healing (19). In osteoblast cell cultures, taurine has been shown to increase ALP and collagen synthesis (21). The findings of our study revealed that ALP levels increased in the 21st day in both groups in line with bone formation.

This study has certain limitations. To create a fracture an open osteotomy technique was utilized which might be different to a closed fracture model. To monitor the oxidative stress, only serum MDA levels were measured; whereas bone tissue levels could have been measured. However, this would be against the reduction principle of animal welfare. Besides MDA, other lipid peroxidation indicators such as superoxide dismutase or glutathione peroxidase could also be measured.

CONCLUSION

The findings of this study supported the theory that oral administration of taurine influenced fracture healing, which was approved by both radiological and histological means. Whether the mechanism of action of taurine on bone healing relies on its antioxidant features or not warrants further study. All in all, as a result of these findings, when treating a fracture, a dietary supplement of taurine could be considered.

Ethics Committee Approval: Ethics committee approval was received for this study from Ege University Local Ethic Committee For Animal Research (Date: 11.04.2008, No: 24).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – E.K.B., A.K.; Design – E.K.B., N.Y., A.K.; Supervision - A.K.; Resources – E.K.B.; Materials – E.K.B.; Data Collection and/or Processing – E.K.B., N.Y., B.D, M.A.; Analysis and/or Interpretation - E.K.B., N.Y., B.D, M.A.; Literature Search - E.K.B.; Writing Manuscript - E.K.B..; Critical Review - A.K.

Acknowledgements: We would like to thank Assistant Prof. Murat Sezak for his contribution to the histological studies, Associative Prof. Timur Kose for the statistical analyses and Dr Ahmet Bicer for his contribution to the editing of the manuscript.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The author declared that this study has received no financial support.

Etik Komite Onayı: Bu çalışma için etik komite onayı Ege Üniversitesi Tıp Fakültesi Hayvan Deneyleri Etik Kurulu'ndan alınmıştır (11.04.2008, No: 24).

Hasta Onamı: Uygulanabilir değil.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Fikir – E.K.B., A.K.; Tasarım – E.K.B., N.Y., A.K.; Denetleme – A.K.; Kaynaklar – E.K.B.; Malzemeler – E.K.B..; Veri Toplanması ve/veya İşlemesi – E.K.B., N.Y., B.D, M.A..; Analiz ve/veya Yorum – E.K.B., N.Y., B.D., M.A.; Literatür Taraması – E.K.B..; Yazıyı Yazan – E.K.B..; Eleştirel İnceleme – A.K.

Teşekkür: Histoloji çalışmalarına katkılarından dolayı Sayın Prof. Dr. Murat Sezak'a, istatistiksel analizler için Doçent Doktor Timur Köse'ye ve makalenin düzenlenmesine katkılarından dolayı Dr. Ahmet Biçer'a teşekkür ederiz.

Çıkar Çatışması: Yazarlar çıkar çatışması bildirmemişlerdir.

Finansal Destek: Yazarlar bu çalışma için finansal destek almadıklarını beyan etmişlerdir.

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