EXPERIMENTAL STUDIES

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The effects of locally applied simvastatin on an experimental mouse femur nonunion model

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Objective: The aim of this study was to assess the effects of locally applied simvastatin on femur nonunions in a mouse model.

Methods: The study included 32 male Wistar albino mice randomly allocated to one of four groups: two control groups (control-4 week [C4w] and control-8 week (C8w)] and two treatment groups (simvastatin-4 week [S4w] and simvastatin-8 week [S8w]). The control groups received dimethylsulfoxide locally injected at a dose of 10 mg/kg/day after surgical intervention for 1 week. Treatment groups received a liquefied form of simvastatin locally to the osteotomy field by injection at a dose of 10 mg/kg/day, starting from the first postoperative day for 1 week. The C4w and S4w groups were sacrificed 4 weeks and the C8w and S8w groups 8 weeks after the end of local treatment. Before sacrifice, intracardiac blood samples were retrieved for biochemical analysis and radiographies were taken. The right femurs of mice were then removed for histopathological evaluation.

Results: There were significant differences between the control and treatment groups when evaluated radiologically. Significantly higher levels of bone-specific alkaline phosphatase and osteocalcin values were found in the treatment groups than in the controls ($p<0.05$).

Conclusion: According to biochemical, radiological and histopathological results, local application of simvastatin appears to produce beneficial effects on the mouse femur nonunion model.

Key words: Femur; mouse; nonunion; simvastatin.

Despite the bone's regenerative and reparative capacity and the progress made in treatment options, approximately 5 to 10% of fractures are associated with impaired healing.[1] Nonunion is a challenging complication for both the patient and physician, often requiring long-term treatment. While the etiology of nonunion is not yet completely known, both systemic and local factors are thought to influence the process.[2]

Statins, which inhibit hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, are widely used as cholesterol-lowering drugs.[3] In addition, statins have antioxidant,[4] anti-inflammatory[5] and vasodilator[6] effects. Statins have been reported to induce osteoblast activity and lead to bone formation, both in tissue cul-

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ture and in rats and mice. Subsequently, evidence for the metabolic effect of statins on bone in vivo has been accumulated.[7,8]

Osteocalcin originates from osteoblastic synthesis and is deposited in the bone or released into the circulation, where it correlates with histological measures of bone formation.[9,10] Serum bone-specific alkaline phosphatase (BAP), which is localized in the membrane of osteoblasts, is the most commonly used marker of bone formation.[11]

The aim of the present study was to investigate the effects of locally applied simvastatin on an experimental mouse femur nonunion model by measuring the changes in serum levels of the bone markers, BAP and osteocalcin, and by evaluating radiological and histopathological changes.

Materials and methods

The study included 32 male Wistar albino mice (mean age: 8 weeks, range: 7 to 9 weeks) weighing 250 (range: 236 to 275) grams. Mice were divided into 4 groups; 2 control groups [control-4 week (C4w) and control-8 week (C8w)] and 2 treatment groups [simvastatin-4 week (S4w) and simvastatin-8 week (S8w)]. Mice were exposed to a 10/14 hour light-dark cycle, kept under normal room temperature and fed by standard pellet food and tap water. The study protocol was approved by the Ethics Committee of Erciyes University Medical Faculty on 8 April 2009 with approval number 09/27.

The simvastatin solution was prepared according to the description by Serin-Kilicoglu and Erdemli.[3] Simvastatin (Zocor© 20 mg film tablet; Merck Sharp & Dohme, White House Station, NJ, USA) was diluted homogenously in 10 mL dimethylsulfoxide (DMSO; 100 ml, 99.5%, D-4540; Sigma-Aldrich Corp., St. Louis, MO, USA), which has no biological effect. Ten mg/kg/day of simvastatin was locally applied to the nonunion area of treatment groups.

The nonunion model was generated according to a procedure described by Garcia et al.[12] The right femurs of all mice were fractured under general anesthesia and retrograde fixation was performed using 1.5-mm Kirschner wires. After fixation, a 1.8 mm gap was made for each mouse by checking with an electronic compass. After periosteal stripping, femurs were fixed using a staple nail. At the end of the 10th week, nonunion was confirmed using radiographs.

The control groups received DMSO locally injected at a dose of 10 mg/kg/day after surgical intervention for one week. The treatment groups received a liquefied form of simvastatin locally to the osteotomy field by injection at a dose of 10 mg/kg/day, starting from the first postoperative day for one week. The C4w and S4w groups were sacrificed at the 4th postoperative week and the C8w and S8w groups at the 8th postoperative week. Anteroposterior and lateral radiographies were obtained before sacrifice and 3 ml of blood was drawn for analysis. After death, the right femurs of the mice were removed as a whole and kept in 10% formaldehyde solution for histopathological examination in all groups.

The Goldberg scoring system[13] was used to evaluate fracture nonunion. Radiographs of each femur were examined by two independent orthopedists and a radiologist. The mean of these three fracture nonunion scores were used for analysis.

A 10-point scale developed by Huo et al.[14] was used for the histological evaluation of fracture healing.

Intracardiac blood samples for biochemical tests were taken from the mice. After separation of plasma, the samples were stored at -70°C until analysis. Serum total cholesterol (TC) was analyzed by an auto-analyzer (Olympus AU2700; Olympus Life and Material Science Europa GmbH, Hamburg, Germany). Serum BAP (IDS, Catalog No: AC-20F1) and osteocalcin (IDS, Catalog No: AC-12F1) were measured using ELISA kits.

Data were analyzed using the SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables, evaluated by the Kolmogorov-Smirnov test, were normally distributed. The one-way ANOVA and Tukey

Table 1. Goldberg scores of treatment and control groups.

Groups	0 point		1 point		2 point		Total		x^2	p
	n	%	n	%	n	%	n	%		
C4w	7	87.5	0	0.0	1	12.5	8	100		
C8w	5	62.5	1	12.5	2	25	8	100		
S4w	3	37.5	2	25	3	37.5	8	100	15.051	0.020
S8w	1	12.5	0	0.0	7	87.5	8	100		
Total	16	50.0	3	37.5	13	40.6	32	100		

tests were used to compare group data. Correlation analyses between variables were made using the Spearman test. The results for qualitative variables were expressed as 'frequency and percentile'. The chi-squared test was used for comparison of qualitative variables. $P<0.05$ was considered as statistically significant. All results were expressed as 'mean with their standard deviation' (mean±SD).

Results

Goldberg scores were significantly different in the treatment groups compared to the controls ($p<0.05$). Table 1 shows the Goldberg scores of all groups. The Goldberg scores of the C4w, C8w, S4w and S8w groups were 2, 5, 8 and 14, respectively. In the C4w group, nonunion was observed in 7 mice (87.5%) and union in one (12.5%). In the C8w group, nonunion was observed in 5 mice (62.5%), evidence of possible union in 1 (12.5%), and union in 2 mice (25%). There were 3 nonunions (37.5%), 2 possible unions (25%), and 3 unions (37.5%) in the S4w group, and 1 nonunion (12.5%) and 7 unions

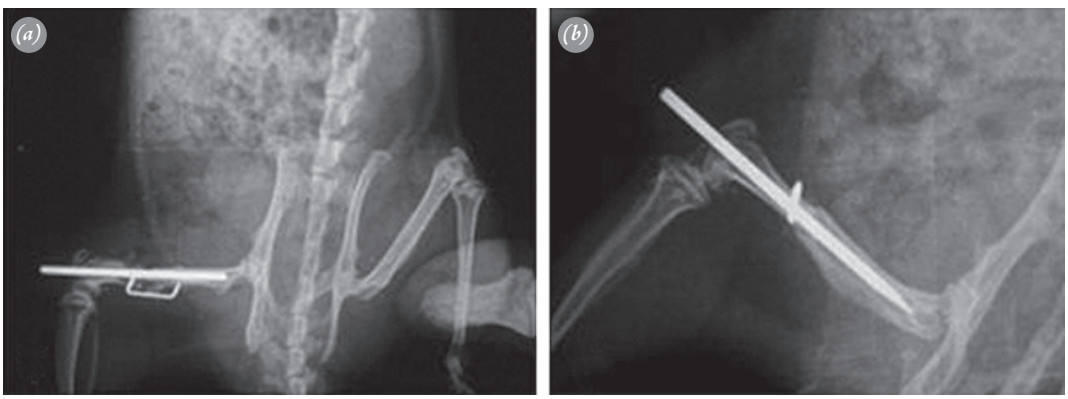

Fig. 1. **(a)** Radiograph of a right femur from the C4w group with a 1.8 mm gap size and stripped periosteum 10 week after surgery. Characteristics of established nonunions can be observed; lack of fracture bridging, absence of callus formation, and rounded bone ends. **(b)** Radiograph of a right femur from the S8w group with characteristically features of union.

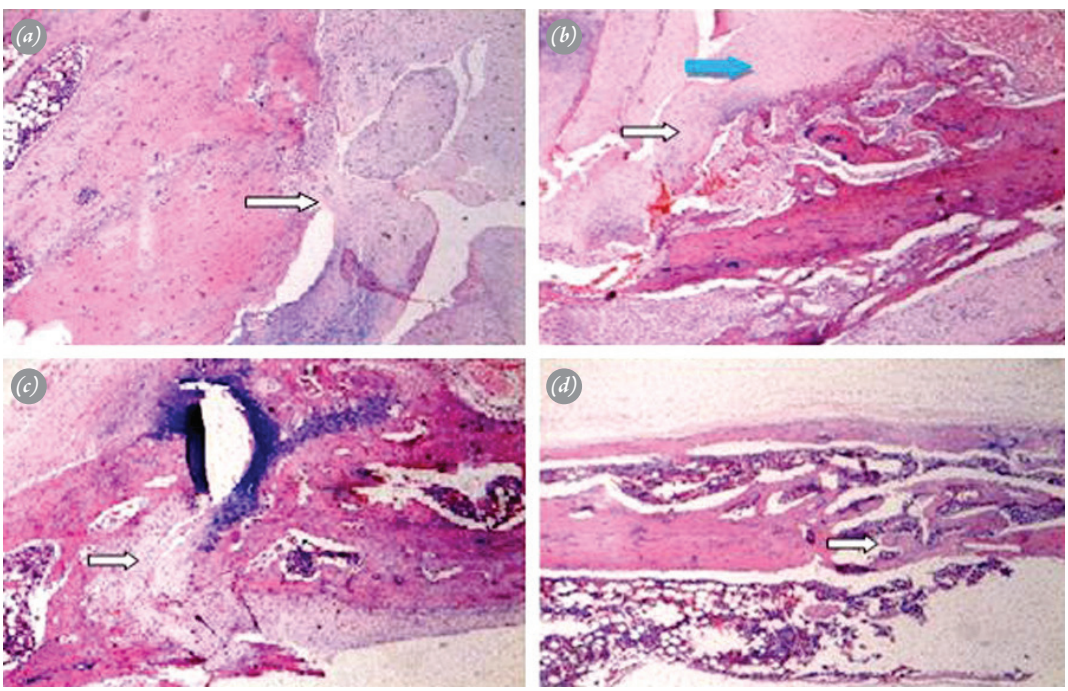

Fig. 2. Photomicrographs of hematoxylin-eosin stained sections of the fracture callus of the study and control groups at the end of the local treatment (magnification: x40). **(a)** Fibrous tissue (white arrow) from the C4w group. **(b)** Small amounts of cartilage tissue (white arrow) and fibrous tissue (blue arrow) from the C8w group. **(c)** Immature bone tissue (white arrow) from the S4w group. **(d)** Healing with mature bone tissue (white arrow) from the S8w group are shown. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Table 2. Results of biochemical measurements.

Parameters	C4w (n=8)	C8w (n=8)	S4w (n=8)	S8w (n=8)	F	p
	Mean±SD	Mean±SD	Mean±SD	Mean±SD		
Total cholesterol	112.3±10.8	112.7±16.1	94.5±9.9	92.2±11.8	6.440	0.002
Bone-specific alkaline phosphatase	12.2±0.8	12.4±2.2	19.7±1.6	22.6±1.1	85.478	<0.001
Osteocalcin	38.6±7.9	41.7±10.3	70.2±8.2	69.0±11.2	25.379	<0.001

(87.5%) in the S8w group.

Nonunion was most common in the C4w group, while union was most commonly seen in the S8w group. Furthermore, when the S4w and S8w groups were compared, union rates increased with a longer treatment period (Fig. 1).

Histopathological scores were significantly different in the treatment groups than the controls ($p<0.05$) (Fig. 2). The histopathological scores of the C4w, C8w, S4w and S8w groups were 31, 35, 55 and 66 respectively. When the treatment and control groups were compared, significant differences were found between the S4w and S8w groups and between the C4w and C8w groups ($p<0.05$). Figure 3 shows the histopathological scores of all groups. In addition, statistically significant positive correlations were found between the radiological and histopathological scores in the S4w and S8w groups ($r=0.878$, $p<0.05$ for S4w group; $r=0.756$, $p<0.05$ for S8w group).

As shown in Table 2, there were significant differences in TC, BAP and osteocalcin levels between the treatment and control groups ($p<0.05$). Total cholesterol levels were significantly lower in the treatment groups than in the control groups ($p<0.05$). There was no statistically significant difference in TC levels between the C4w and C8w groups ($p=0.876$) or between the S4w and S8w groups ($p=0.903$).

Plasma BAP levels were significantly higher in the treatment groups than in the controls ($p<0.05$). There was no significant difference between the C4w and C8w groups in BAP levels ($p=0.898$), while the BAP levels of the S8w group were higher than those of the S4w group ($p<0.05$).

Plasma osteocalcin levels were significantly higher in the treatment groups than in the control groups ($p<0.05$) (Table 2). There was no statistically significant difference in osteocalcin levels between the C4w and C8w groups ($p=0.917$), or between the S4w and S8w groups ($p=0.895$).

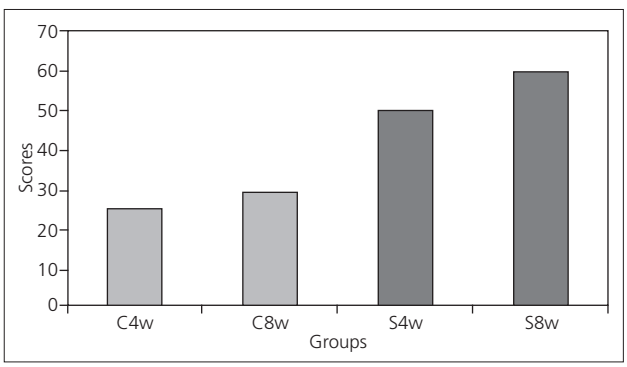

Fig. 3. Results of histopathological evaluations.

Discussion

This experimental study demonstrated the favorable effects of simvastatin on fracture healing in an experimental setting, as evidenced by radiographic, biochemical and histopathological findings.

Recent studies have suggested the positive effects of statins on bone healing. In one previous experiment, simvastatin was injected subcutaneously to the fracture site in rats and increased callus area enhanced fracture strength at 2 weeks after fracture.[15] Simvastatin was directly applied to the femur fracture area in mice in another study. This study found a dramatic positive effect on biomechanical parameters of fracture healing by simvastatin.[16] Fukui et al. suggested that local administration of low-dose simvastatin-conjugated gelatin hydrogel could be a promising therapeutic strategy for fracture repair in clinical settings.[17] In the present study, a dose of 10 mg/kg/day was selected for local administration. Our in vivo study showed that simvastatin administration promoted radiographic fracture repair and had a positive effect on histopathological and biochemical scores.

Ayukawa et al. reported that local application of simvastatin promotes bone repair in rats through the suppression of osteoclasts and the enhancement of osteoblasts at bone-healing sites by stimulating bone morphogenetic protein-2 (BMP-2) mRNA.[18] Wang et al. reported that statin use by elderly patients causes a reduction in the risk of hip fracture.[19] In another study, Edwards et al. demonstrated a significant increase in bone mineral density associated with statin use in postmenopausal women.[20]

Although most observational studies have shown the positive effects of statins on bone mass and fracture risk,[21,22] some controversial data has been reported.[23] Van Staa et al. declared the use of statins at dosages prescribed in clinical practice was not associated with a reduction in risk of fracture.[24] LaCroix et al. reported that statin use did not improve fracture risk or bone density in postmenopausal women and the cumulative evidence did not warrant use of statins to prevent or treat osteoporosis.[25] In another study reporting the opposite view, von Stechow et al. reported that statins had no clear effects on bone formation *in vivo*.[26]

Statins stimulate bone formation when given in large doses or by prolonged infusion. This is mainly due to first-pass metabolism of these drugs in the liver when such drugs are administered orally. Much greater doses would be toxic for the liver and muscles.[27] In a study by Garrett et al., statins showed a BMP-2 gene stimulating effect only when administered locally.[27] Gutierrez et al. compared the effects of oral and local lovastatin on the bone formation of rats and reported that local application of statins produced greater beneficial effects on bone formation than oral administration.[7] For this reason, simvastatin was administered locally to tissue in close proximity of the fracture in the present study.

Direct radiography, which is easy to practice and simple to evaluate, is a noninvasive method for the assessment of fracture healing. The present study used the radiological classification system developed by Goldberg et al.[13] in 1985 and adopted by many authors. There was a statistically significant difference between the Goldberg scores of the study and control groups and between the S4w and S8w groups. This was confirmed by the findings of the biochemical and histopathological examinations. In the present study, a scale developed by Huo et al.[14] was used for the histological evaluation of fracture healing. While more fibrous tissue and small amounts of cartilage tissue were seen in the control groups, immature and mature bone tissue were seen in the simvastatin groups. When the findings of the study and control groups were evaluated together, statistically significant and strong positive correlations were found between the radiological and histopathological scores in the S4w and S8w groups.

Biochemical parameters have been measured by many authors to prove both the systemic and bone formation stimulating effects of locally administered simvastatin. Wang et al. showed that simvastatin locally applied to rats with tibial fractures decreased serum TC and increased BAP level.[14] Gutierrez et al. reported increased osteocalcin levels in rats with the use of transdermal lovastatin.[7] In contrast to this study, Rosenson et al. declared that statins had no effect on bone formation markers, osteocalcin or BAP.[28]

In the present study, the systemic efficiency of simvastatin was shown by measuring serum TC levels. Total cholesterol levels were found to be lower in the treatment groups than in the control groups. Plasma BAP and osteocalcin levels were significantly higher in the treatment groups than in the controls.

In conclusion, when radiological, histopathological and biochemical findings are evaluated together, simvastatin appears to improve the healing of nonunions. Current experimental evidence suggests the potential beneficial effects of simvastatin on fracture healing. In addition, the substance has been shown to be safe in experimental studies. We believe that simvastatin has the potential to be used locally in fracture healing in the future. Further studies examining the effect of this substance in clinical settings are warranted.

Conflicts of Interest: No conflicts declared.

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