

Research Article

Effect of Simvastatin Administration on ALP (Alkaline Phosphatase) Level in Wistar Rat's Femur Fracture

Pengaruh Pemberian Simvastatin terhadap Kadar ALP (Alkali Fosfatase) pada Patah Tulang Femur Tikus Wistar

Hery Susilo¹, Edi Mustamsir², Wanda Gusta Rai^{3*}

^{1,3}Department of General Surgery Faculty of Medicine Universitas Brawijaya Malang

²Department of Orthopedic and Traumatology Faculty of Medicine Universitas Brawijaya Malang

ABSTRACT

Metabolism of the bone healing process can be monitored by Bone formation marker, such as alkaline phosphate (ALP). Simvastatin is known to increase bone marker formation in the repair phase, but its effects on the ALP level have not been known yet. This study aimed to determine the effect of oral simvastatin administration on the expression of ALP level in femur fracture healing in a rat model of femur fracture. The research method was experimental post-test only using 18 *Rattus norvegicus* rats which were divided into 3 groups, namely the control group for femur fracture (Control), femoral fracture group and giving simvastatin 0.36 mg/day for 2 weeks (Treatment I), and femur fracture group and administration of simvastatin 0.36 mg/day for 4 weeks (Treatment II). ALP levels were measured at 0, 2, and 4 weeks. The results showed an increase in ALP levels in the simvastatin treatment group compared to the control group ($p < 0.05$). It can be concluded that the administration of simvastatin increased ALP levels in rat femoral fractures.

Keywords: Alkaline phosphate, femur fracture, simvastatin

ABSTRAK

Metabolisme proses penyembuhan tulang dapat dimonitor dengan *bone marker formation*, salah satunya *alkaline phosphate* (ALP). Simvastatin diketahui dapat meningkatkan *bone marker formation* pada fase reparasi namun efeknya terhadap kadar ALP belum diketahui. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian simvastatin oral terhadap kadar ALP pada proses penyembuhan tulang femur tikus model fraktur. Metode penelitian adalah *experimental post-test only* dengan menggunakan 18 ekor tikus *Rattus norvegicus* yang dibagi dalam 3 kelompok yaitu kelompok kontrol fraktur femur (*Control*), kelompok fraktur femur dan pemberian simvastatin 0,36mg/hari selama 2 minggu (*Treatment I*), dan kelompok fraktur femur dan pemberian simvastatin 0,36mg/hari selama 4 minggu (*Treatment II*) kemudian kadar ALP diukur pada minggu ke-0, ke-2, dan ke-4. Hasil penelitian menunjukkan peningkatan kadar ALP pada kelompok perlakuan simvastatin dibandingkan dengan kelompok kontrol ($p < 0,05$). Dapat disimpulkan bahwa pemberian simvastatin meningkatkan kadar ALP pada patah tulang femur tikus.

Kata Kunci: Alkaline phosphate, fraktur femur, simvastatin

Correspondence: Wanda Gusta Rai. Department of Urology Faculty of Medicine Universitas Brawijaya Malang, Jl. Veteran Malang Tel. 081230001521 Email: wanda080190@gmail.com

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INTRODUCTION

Fracture is a condition of a loss in the continuity of bone, joint cartilage, epiphyseal cartilage, either total or partial. Most fractures occur due to bone failure to withstand stress, especially bending, twisting, and tensile stresses. The most common cause of fractures is due to accidents, whether work, traffic, or other accidents. The World Health Organization (WHO) noted that between 2011 and 2012, 5.6 million people died, and 1.3 million people suffered fractures due to traffic accidents (1).

The metabolism of the bone healing process can be monitored by bone formation markers. Osteoblasts are cells derived from the mesenchyme, which is the main producer of proteins present in the extracellular matrix of bones. Osteoblasts control the mineralization process of the extracellular matrix (2,3).

Alkaline Phosphatase (ALP) will increase in the repair phase when there is a synergy to increase the osteoblast levels in the callus formation phase. Expression of ALP mRNA increases in 10 to 14 days after the fracture or repair phase. In this period, fibrous tissue forms new bone, and osteoblasts form trabecular bone. This high ALP is not found during the inflammatory phase and hematoma during the fracture healing process (3).

Many researchers tried to add substances, such as simvastatin, to speed up the healing process in bones. Statins are coenzyme A 3-hydroxy-3-methylglutaryl reductase inhibitors and are widely used as antihypercholesterolemic drugs. Simvastatin has an effect on osteoblastic bone formation. This drug induces odontoblast cell differentiation and deposition of mineralized matrix by activating the ERK1/2 and Smad1/2/3 signaling (4), which are associated with increased bone morphogenetic protein (BMP)-2 expression. Simvastatin could increase osteoblast and bone marker expressions, such as BMP, osteocalcin, osteopontin, and alkaline phosphatase (ALP) in vitro for three days, but its effect after the first week has not been studied (5). This study was conducted to prove the effect of simvastatin on ALP levels in rats by measurement in week 2 and week 4 in vivo.

METHOD

This study was conducted through a laboratory

experiment using a post-test only with control group research design. The research was conducted at the Surgery Department of the Faculty of Medicine Universitas Brawijaya and Gamma Scientific Laboratory from 10 October 2020 to 30 November 2020. All experiment protocol were approved by Medical and Health Research Ethics Unit, Faculty of Medicine Brawijaya University/ Saiful Anwar General Hospital.

This experiment used 18 male rats (*Rattus norvegicus* Wistar strain) aged 3 months and purposely fractured in the middle 1/3 fracture of the femur, which were randomly divided into three groups namely Control, Treatment I, and Treatment II groups. Control group was only given normal saline 0.9% 2ml, Treatment I group was given Simvastatin orally 0.36mg/day for 2 weeks, and Treatment II was given Simvastatin orally 0.36mg/day for 4 weeks (Figure 1A). Several rats of treatment group II were sacrificed in weeks 0, 2, and 4 for histological analysis by staining with hematoxylin eosin. Every day, rats were given Comfeed PAR-S and water ad libitum.

Blood serum was collected by laboratory staff at three different times: weeks 0, 2, and 4 on the rat tail vein as much as 0.5cc using a wing needle in a sterile manner (Figure 1B). Then the blood samples were checked for ALP analysis. ALP activity determination was done by the enzymatic reaction based on ALP Dyasis® protocol and measured spectrophotometrically at 405 nm. Histological analysis was performed as procedure in elsewhere.

Data were exhibited as mean \pm Standard Deviation. One way ANOVA or Kruskal Wallis followed by post hoc test was conducted to set the level of significance (p -value of < 0.05). Statistical analysis was conducted using SPSS V.21.0 for Windows software.

RESULT

Differences in ALP Levels among Groups at Each Measurement Time

The data (Table 1) exhibit that all groups were start at the comparable ALP level and increase by the time. The lowest ALP level was in the week 0 and the highest ALP level was in the week 4. Treatment II group showed the highest ALP level in the week 4 (Figure 2).

The Kruskal Wallis test results showed that the mean level

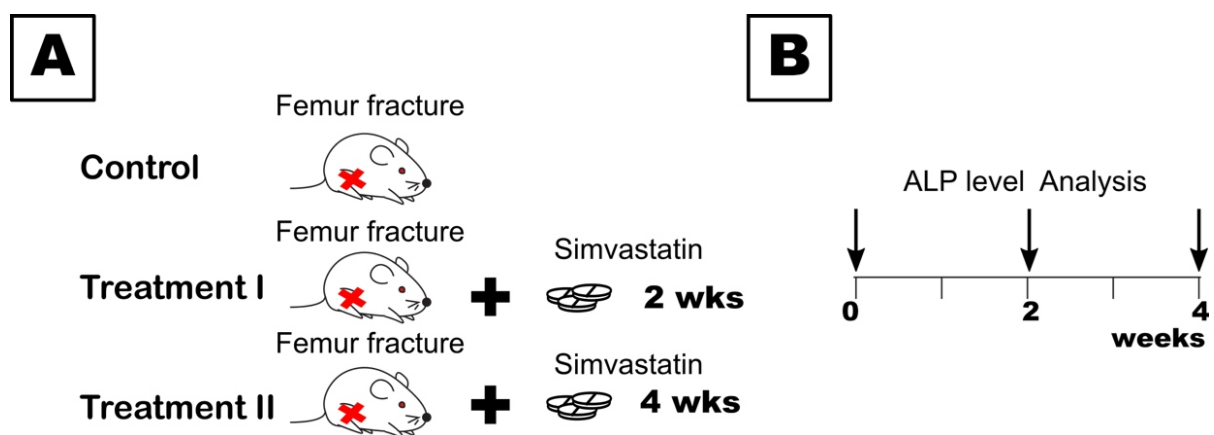


Figure1. Schematic experimental procedure. A. rats were divided into 3 groups consist of 1 control group and 2 treatment groups B. ALP level analysis was performed in weeks 0, 2, and 4.

of ALP between the control and treatment groups in week 0 was not significantly different ($p=0.290$). In week 2, there was a significant difference between the control and treatment groups ($p=0.003$). The Mann-Whitney test showed a significant difference between the control group and the treatment groups 1 and 2, but there was no significant difference between treatment groups 1 and 2.

The one-way ANOVA test results indicated that the mean level of ALP between treatment and control groups in week 4 ($p=0$) was significantly different. Based on the independent t-test displayed in the mean table, there is a significant difference between the control and treatment 1 and 2 groups and also between treatment groups.

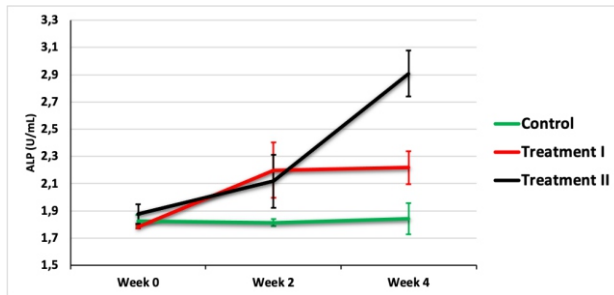


Figure 2. ALP levels Analysis. ALP level was increased over the time and reach the highest ALP level in week 4. Data were shown as mean \pm S.D.

Differences in ALP Levels at Weeks 2 and 4

The results showed the mean level of ALP in the control group on weeks 2 and 4 was 1.783 ± 0.03 U/mL and 1.816 ± 0.08 U/mL, respectively, the treatment group 1 on week 2 was 2.198 ± 0.20 U/mL and 2.217 ± 0.19 U/mL on week 4, and the treatment group 2 in week 2 was 2.118 ± 0.12 U/mL and 2.908 ± 0.16 U/mL on the 4th week. The results of the Wilcoxon test show that the mean ALP levels of the control group and treatment 1 did not differ in week 2 and week 4. A different result was found in treatment group 2 that there was a significant difference between week 2 and week 4 ($p=0.028$; $p<0.05$).

Table 1. Differences in ALP levels among groups at each measurement time

Group	Average ALP Levels (U/mL)			P-value (Between week 2-4)
	Week 0	Week 2	Week 4	
Control	1.825 ± 0.01	1.783 ± 0.03	1.816 ± 0.08	0.917
Treatment 1	1.791 ± 0.01	2.198 ± 0.20	2.217 ± 0.19	0.753
Treatment 2	1.876 ± 0.11	2.118 ± 0.12	2.908 ± 0.16	0.028
p-value (Among groups)	$p=0.290$	$p=0.003$	$p=0.000$	

Note: Significance at $p<0.05$

To convince about bone formation in Treatment II group, we performed histology analysis in the week 0, 2, and 4 (Figure 3). The bone formation can be clearly seen in the week 2 and 4.

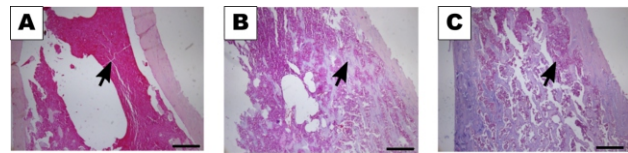


Figure 3. Histology of bone formation in Treatment II group. A. Representative image of femur fracture in week 0. B. Representative image of bone formation in week 2. C. Representative image of bone formation in week 4. Fracture line were indicated by arrow. Scale bar, 100 μ m

Note:

A. Representative image of femur fracture in week 0.
B. Representative image of bone formation in week 2.
C. Representative image of bone formation in week 4.
Fracture line were indicated by arrow. Scale bar, 100 μ m

DISCUSSION

The study results showed that the administration of simvastatin gave higher ALP levels in weeks 2 and 4. Longer administration (week 4) also shows higher ALP levels.

Alkaline Phosphatase (ALP) plays a role in the mineralization process to prepare an alkaline atmosphere in formed osteoid tissue so that calcium can easily be deposited in the tissue. This enzyme also causes increased phosphate concentration, thus calcium-phosphate bonds are formed in hydroxyapatite crystals that settle in the bones (6).

Serum ALP is derived from the liver, bone, intestine, spleen, kidneys, and placenta. Most adult serum ALP comes from the liver and bone. ALP derived from bone is synthesized by osteoblast, so it reflects the osteoblast activity during bone formation (7). ALP in the bone is a good marker of bone formation because of its ease of measurement, cost efficiency, and high specificity in detecting small changes in bone formation. However, cross-reactivity with hepatic ALP is one of the drawbacks of the bone ALP test (8). ALP levels are known to be affected by triglyceride levels due to lipid oxidation activity. Simvastatin is an antilipemic that can reduce triglyceride levels. Thus, further research on the effect of simvastatin on ALP needs to be conducted.

In hyperlipidemia conditions, lipid metabolism disorders occurred. The presence of lipid metabolism disorders can cause a high level of oxidized lipids, which can lead to upregulation of peroxisome proliferator-activated receptor- γ (PPAR- γ). PPAR- γ is a special receptor for regulating adipocyte differentiation and proliferation. Besides, it also controls lipid metabolism and inflammation. Activated PPAR- γ can stimulate adipocyte differentiation but also suppress osteoblast differentiation (9).

This present study indicated that the administration of simvastatin increased ALP levels. Administration of simvastatin is intended to inhibit the formations of cholesterol and triglycerides by inhibiting HMG-CoA reductase to reduce triglycerides and cholesterol levels. This process decreases the fat content, thereby decreasing the lipid oxidation process. This reduction in lipid oxidation decreases PPAR- γ , so it increases osteoblast differentiation, which can increase ALP levels (10).

Statins are known to inhibit farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and estrogen receptors (ER) that play a role in bone anabolism. FPP and

GGPP are known to inhibit osteogenic differentiation in MC3T3-E1 cells; thus, protein is considered to inhibit bone formation. Statin administration can inhibit these proteins, so it increases bone formation. Statins are also known to increase osteoprotegerin and osteoblast differentiation characterized by elevated ALP and osteocalcin (11).

Statins modulate osteoclastogenesis via the OPG/RANKL/RANK signaling pathway. A previous study found that statin increased OPG mRNA expression but decreased RANKL mRNA expression (12). Ayukawa *et al.*, treated artificial fractures in mice with simvastatin for 3 consecutive days. On day 5 after simvastatin injection, RANKL expression was suppressed and there were fewer osteoclasts, while the area of newly formed bone was larger than that of the control group (13). Han *et al.*, found a similar result that simvastatin inhibited osteoclast activity and stimulated new bone formation in the periodontal tissue of mice (14).

The results of this study were in line with the research conducted by Jadhav *et al.*, that the administration of statins for 2 weeks increased ALP levels in rats (4). However, these results differed from the study by Stein *et al.*, that simvastatin administration doses of 40 mg and 80 mg for 12 weeks decreased ALP levels in women and men (15). Research by Rosenson *et al.*, also showed that administering simvastatin 80 mg for 8 weeks decreased serum ALP levels in humans (16). The other result was also

shown by Hsia *et al.*, that simvastatin administration for 12 weeks in women with osteopenia did not change ALP levels compared to controls.

In this study, ALP levels were significantly lower when simvastatin was given for 4 weeks. This decrease can be caused by the longer duration of simvastatin that extends the process of lipid oxidation inhibition. The inhibition of lipid oxidation decreases PPAR- γ thereby increasing the differentiation of osteoblasts. Research by Hsia *et al.*, proved no difference between simvastatin administration for 6 weeks and 12 weeks in women with osteopenia. The differences in the results of this study were thought to be due to differences in the subject of research and the difference between the duration of simvastatin administration, resulting in different outcomes.

This study proves that the administration of simvastatin increases ALP levels on Wistar rats' femur fracture treated conservatively using CAST. Increasing the duration of simvastatin administration increases rats' ALP levels. Further research using human research subjects with equal simvastatin doses is needed. Further study also needs to be done by measuring bone marrow density, the number of osteocytes, and osteoclasts.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest either in the research or writing processes.

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