


Conventional Low Intensity Pulsed-Ultrasound Therapy Increases Osteoblast, Serum Alkaline Phosphatase, and Serum Calcium Levels in Fracture Healing Process

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ABSTRACT

Introduction: Application of ultrasound waves to improve bone healing generally use specific bone stimulator equipment not available in Indonesia. Frequency and duration of therapy from previous studies are very difficult to apply in clinical practice. This study aims to observe the therapeutic effect of conventional low-intensity pulsed-ultrasound to osteoblast, alkaline phosphatase, and serum calcium levels. **Method:** Thirty six male white rats were divided into three groups (control, USD 5x/week, and USD 3x/week). Tibial fracture in ultrasound groups were treated 3x/week and 5x/week with ultrasound waves (1 MHz, pulsed mode, 20% of duty cycle, intensity of 0.2 W/cm^2, duration 10 minutes, stationary) for 3 weeks. Callus tissue and blood from all animals were assessed quantitatively using histological and biochemical analyses. **Result:** Significant differences ($p<0.05$) in the average number of osteoblasts, level of alkaline phosphatase, and serum calcium among all three groups. **Conclusion:** Conventional low intensity pulsed-ultrasound either 5x/week or 3x/week improve bone healing process.

Keywords: Alkaline phosphatase, calcium, fracture, osteoblast, ultrasound

ABSTRAK

Pendahuluan: Penggunaan gelombang ultrasonik untuk memperbaiki penyembuhan tulang umumnya menggunakan alat stimulasi khusus yang tidak tersedia di Indonesia. Frekuensi dan durasi terapi penelitian sebelumnya sulit diterapkan dalam praktik klinis. Tujuan penelitian ini adalah untuk membandingkan efek stimulasi gelombang ultrasonik menggunakan alat terapi konvensional *ultrasound diathermy* (USD) antara kelompok kontrol, kelompok perlakuan USD 5x/minggu dan 3x/minggu terhadap jumlah osteoblas, kadar alkali fosfatase, dan kalsium serum. **Metode:** Sejumlah 36 ekor tikus putih jantan dibagi tiga kelompok (kontrol, USD 5x/minggu, dan USD 3x/minggu). Fraktur tibia pada kelompok *ultrasound* diterapi 3x/minggu dan 5x/minggu dengan gelombang ultrasonik (1 MHz, *pulsed-mode*, 20% *duty cycle*, intensitas 0,2 W/cm^2, durasi 10 menit, stasioner) selama 3 minggu. Jaringan kalus dan darah semua hewan dinilai secara kuantitatif dengan analisis histologi dan biokimia. **Hasil:** Ada perbedaan signifikan ($p <0{,}05$) jumlah rata-rata osteoblas, kadar alkali fosfatase, dan kalsium serum antara ketiga kelompok. **Simpulan:** Terapi konvensional dengan *pulsed-ultrasound* intensitas rendah frekuensi 5x/minggu ataupun 3x/minggu memperbaiki proses penyembuhan tulang. **Indrayuni Lukitra Wardhani, I Ketut Gede Agus Budi Wirawan, I Putu Alit Pawana, Andriati, Patricia Maria. Terapi Konvensional *Low Intensity Pulsed-Ultrasound* Meningkatkan Osteoblas, Kadar Fosfatase Alkali Serum, dan Kalsium Serum pada Proses Penyembuhan Fraktur**

Kata kunci: Alkali fosfatase, fraktur, kalsium, osteoblas, ultrasonik

INTRODUCTION

The expected outcome of fracture therapy is bone healing, defined as the functional stage of bone regeneration after a trauma, enabling the bone to acquire its anatomical and load-carrying properties.[1] Several strategies have been developed to improve bone healing, including the use of physical modalities of ultrasound waves.

According to Wolff's law, ultrasonic stimulation generates micro-mechanical forces and tension on the fracture site, resulting in accelerated bone formation.[2,3] The direct action of low-intensity ultrasound on mechanoreceptors is probable, but it can also act through the release of osteoblast agonists, inducing their proliferation and differentiation, and the release of prostaglandins through activation of P2X7 receptor in bone cells, allowing inflow of ions, such as calcium.[3] Osteoblasts secrete large quantities of ALP, which is involved in the process of bone matrix formation and its mineralization.[4]

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Research on the effects of ultrasound waves on bone healing generally uses a bone stimulator called SAFHS (sonic accelerated fracture healing system) or LIPUS (low-intensity pulsed ultrasound). The common applied dose and used in most studies are: frequency of 1.5 MHz, intensity of 30 mW/cm^2, 20% duty cycle, and 20 minutes once a day at home. Unfortunately, this equipment is not available in Indonesia and more costly than conventional Ultrasound Diathermy (USD) therapy.[3,5,6] Several studies have examined the effects of therapeutic ultrasound using the conventional USD. Fontes-Pereira, *et al*, has proven that therapeutic ultrasound given for 10 minutes, once a day, over five consecutive days with two days interval, for 25 sessions, can accelerate fracture healing. The usual dose of USD therapy in Indonesia's clinical practice is two to three times a week for 5-10 minutes. Research using conventional USD in fracture healing is also lacking.[3,6-8] Frequency and duration of therapy from prior studies is difficult to apply clinically because of some technical and financial problems.

To elucidate the effect of therapeutic ultrasound, histological osteoblasts cell analysis was conducted and biochemical analysis was conducted on alkaline phosphatase activity (indicates osteoblast activity that determines bone formation or reabsorption) and serum calcium (indicates bone matrix synthesis process).[3]

The aim of this study is to compare the effect of low-intensity pulsed-ultrasound 5 times a week and 3 times a week to osteoblasts, level of alkaline phosphatase and serum calcium, and tibial fracture healing process of *Rattus norvegicus*. The originality of the present study is to investigate the effect of therapy session in common clinical practice in Indonesia.

MATERIALS AND METHOD

A total of thirty six male Wistar rats, 3-4 month old, body weight 150-200 g, were selected for this study and maintained in a controlled environment. The animals were randomly divided into three groups (control, USD 5x/week, and USD 3x/week). Each group consisted 12 rats that underwent the same fracture induction procedure.

Prior to fracture induction, the rats were anesthetized with intraperitoneal ketamine (75-100 mg/kg). To attain standardized experimental fractures, a simple and portable apparatus was made based on Bonnarens & Einhorn design according to 'three point bending' principal, with some modification (**Figure 1**).[9,10] This apparatus produces a closed fracture of middle third of right tibia with minimal soft tissue damage.[9] Fractures are then immobilized with plastic splint and plaster to ease treatment.

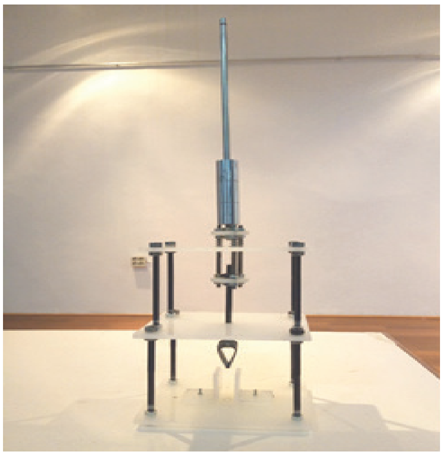


Figure 1. The fracture apparatus

The ultrasound device is portable USD equipment with main power: 120-240 VAC ~ 50/60 Hz, electrical safety class: class 1 Type B, dimension: 33 x 29 x 16.3 cm, weight: 2.3 kg, transducer size: 5 cm^2, BNR (max): 5:1, ERA: 4.0 cm^2. All rats were submitted to bone fracture, control group (n=12) did not receive ultrasound stimulation. Ultrasound therapy for both treatment groups commenced on 48 hours after fracture induction (frequency 1 MHz, duty cycle 20%, intensity 0.2 W/cm^2, duration 10 minutes). Group USD 5x/week (n=12) receive five consecutive days of low-intensity pulsed ultrasound stimulation and two days interval, for three weeks (a total of 15 therapy sessions). Group USD 3x/week (n=12) receive a low-intensity pulsed ultrasound stimulation on alternating days for three weeks (a total of 9 therapy sessions). The transducer was stationary and positioned perpendicular to the fracture area by bladder technique using water-filled glove coated with thin gel on both sides.

Levels of blood alkaline phosphatase (ALP) and serum calcium (SC) were determined after 12 hours of fasting on the 23rd day after fracture. All rats from three groups were terminated within 24 hours after completed 3 weeks of treatment. After anesthesia, rats were submitted to exsanguination through cardiac puncture (5 mL).

The right hind limb was dissected, soft tissue including skin and muscles were removed until the tibia was totally exposed; the tibias were decalcified in a solution and subsequently processed in paraffin. Paraffin blocks were sectioned longitudinally and histological sections were prepared and stained with Hematoxilyn Eosin (HE) for quantitative count of osteoblasts. Histological analysis were performed by expert and blind to group allocation

Shapiro-Wilk normality test was used to study all variables and One-way ANOVA (Analysis of Variance) test was used for comparisons between and within groups. Statistical analysis was performed using SPSS (version 19.0, IBM Corporation,Armonk, NY, USA). The level of significance was $\alpha = 0.05$, with a confidence interval of 95 %. The data are presented as

Table 1. Mean and standard deviation of the dependent variables.

Variable	Groups	N	Mean ± SD	*p* value
Osteoblasts	Control	12	31.50 ± 6.69	0.000
	USD 5x/week	10	92.66 ± 8.13	
	USD 3x/week	12	66.75 ± 9.34	
ALP	Control	12	166.08±34.07	0.010
	USD 5x/week	10	217.60±41.55	
	USD 3x/week	12	189.92 ± 34.89	
SC	Control	12	10.27 ± 0.39	0.013
	USD 5x/week	10	10.33± 0.27	
	USD 3x/week	12	10.65 ± 0.26	

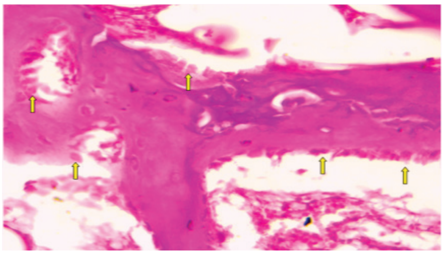
mean ± SD.

RESULTS

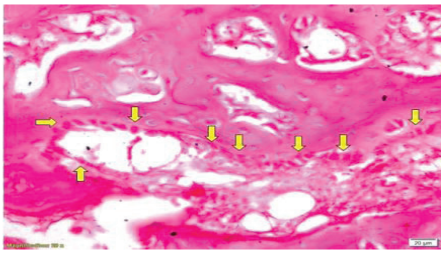
Two animals from group USD 5x/weeks died during first week of intervention. Only 34 samples were available for statistical analysis. No significant difference in body weight among the three groups prior to intervention (p >0.05).

Results showed that ultrasound stimulation significantly increase the amount of osteoblasts. There were significant differences (p=0.000) of the average number of osteoblasts between control group and group of USD 5x/week, between control group and group of USD 3x/week, and between group of USD 5x/week and USD 3x/week (**Table 1** and **Table 2**).

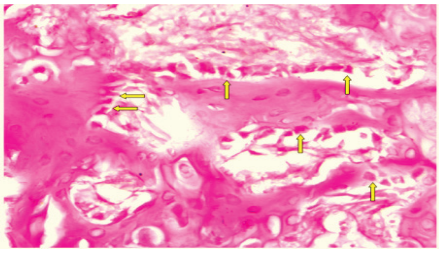
The USD 5x/week and 3x/week groups presented higher levels of ALP and SC than control group (p=0.010 and p=0.013, respectively) (**Table 1**). Using multiple comparisons test, ALP level in USD 5x/week group is significantly different from control group. The SC level in USD 3x/week group differs significantly from control and USD 5x/week group. The ALP level is not significantly different between control and USD 3x/week group, and between USD 5x/week and USD 3x/week group. The mean difference of SC is non-significant between control and USD 5x/week group.



Control



USD 5x/week



USD 3x/week

Figure 2. Presence of osteoblast in woven bone tissue (H.E. ±400X)

DISCUSSION

This study demonstrated, through histological analyses, that low-intensity pulsed-ultrasound significantly increases the amount of osteoblasts which means increasing bone formation. This result is in accordance with previous study by Fontes-Pereira, *et al*, stating that low-intensity pulsed-ultrasound is a good stimulator of the different cells of the osseus systems, accelerating the healing of clinical fracture and increasing bone formation through osteoblast activity.[3] The direct effect of low-intensity ultrasound stimulation on osteoblast functionalization is characterized by accelerated bone nodule formation, calcification and osteocalcin band formation in osteoblast culture.[12,13]

Histological analysis also suggests that increase of osteoblasts is proportional to the increased therapy sessions. Wolff's law stated that stress or mechanical forces is not only alter the architecture and bone mass, but also alter bone structure in response to the mechanical load. The greater energy received to a certain tolerance limit, the greater accelerating effect of callus formation.[14-16]

Biochemical analysis was performed on alkaline phosphatase and serum calcium. These markers were used to evaluate the process of bone formation since calcium is a component of the bone matrix and alkaline phosphatase activity was noted in osteoblastic formation. The small temperature increase by ultrasonic waves has a repercussion

Table 2. Multiple comparisons test of the dependent variables.

Dependent Variable	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
OSTEOBLAST	CONTROL	USD 5X/week	**-61.16700***	3.48017	**.000**	-68.2649	-54.0691
		USD 3X/week	**-35.24833***	3.31821	**.000**	-42.0159	-28.4808
	USD 5X/week	CONTROL	**61.16700***	3.48017	**.000**	54.0691	68.2649
		USD 3X/week	**25.91867***	3.48017	**.000**	18.8208	33.0165
	USD 3X/week	CONTROL	**35.24833***	3.31821	**.000**	28.4808	42.0159
		USD 5X/week	**-25.91867***	3.48017	**.000**	-33.0165	-18.8208
ALP	CONTROL	USD 5X/week	**-51.51667***	15.70509	**.003**	-83.5474	-19.4859
		USD 3X/week	**-23.83333**	14.97421	**.122**	-54.3734	6.7068
	USD 5X/week	CONTROL	51.51667*	15.70509	.003	19.4859	83.5474
		USD 3X/week	**27.68333**	15.70509	**.088**	-4.3474	59.7141
	USD 3X/week	CONTROL	23.83333	14.97421	.122	-6.7068	54.3734
		USD 5X/week	-27.68333	15.70509	.088	-59.7141	4.3474
SC	CONTROL	USD 5X/week	-.06283	.13469	.644	-.3375	.2119
		USD 3X/week	**-.38333***	.12842	**.005**	-.6452	-.1214
	USD 5X/week	CONTROL	.06283	.13469	.644	-.2119	.3375
		USD 3X/week	**-.32050***	.13469	**.024**	-.5952	-.0458
	USD 3X/week	CONTROL	**.38333***	.12842	**.005**	.1214	.6452
		USD 5X/week	**.32050***	.13469	**.024**	.0458	.5952

*. The mean difference is significant at the 0.05 level.

on the activity of some enzymes, namely, matrix metalloproteinase-1 and collagenase. Accordingly, ultrasound can serve to effectively reestablish or normalize metabolic temperatures in the tissue healing regions; it also increases the activity of ALP and SC.[2,3]

In a study by Komnenou, *et al*, (2005)[17] on dogs, and by Ajai, *et al*, (2013)[4] on human patients, serum alkaline phosphatase (ALP) activity was determined throughout the healing process of long bone diaphyseal fractures. In both studies, serum ALP activity remained within reference limits during the entire postoperative period in patients that developed a non-union process, probably indicating a suppression of osteoblastic activity. Ajai, *et al*, (2013)[4] also observed significantly higher levels of serum ALP activity in normal bone union group as compared with delayed healing group.

Intervention with low-intensity pulsed ultrasound in our study (1 MHz, intensity of 0.2 W/cm^2 with a duty cycle of 20%, applied in stationary form during 10 minutes in the fracture region, for 3 weeks) improves bone formation and mineralization, confirmed by histological and biochemical analysis. The intensity of 0.2W/cm^2 was used as it is commonly found in commercial ultrasound equipment in clinical practice; a typical therapy machine is not able to set power densities at below 0.1 W/cm^2, while specific equipment uses 30mW/cm^2 and 3 to 4 times more costly.[3] Low intensity ultrasound wave can be used to stimulate cell activity, while high intensity stimulation can otherwise lead to cell damage. Intermittent or pulsed stimulation gives the cell a chance to rest and adapt, while continuous stimulation leads to tissue heating and potentially inhibits cell activity. The combination of optimal frequency, intensity, interval and duration of mechanical stimulation will improve the quality of bone matrix formation.[6,12,18,19]

This study also showed that stimulation of low-intensity pulsed-ultrasound three times a week (dosage of modalities therapy in general practice) is sufficient to provide a significant effect of increasing osteoblasts and calcium levels. The use of conventional ultrasound device with lesser frequency of therapy could mean a reduction in treatment costs, making this therapeutic modality more accessible to the general population. This result also can be a basis for further study of therapeutic ultrasound application with conventional USD apparatus in improving bone healing in humans and ultimately be a solution in clinical practice.

CONCLUSION

Conventional low intensity pulsed-ultrasound given either 5x/week or 3x/week significantly increase osteoblasts, serum alkaline phosphatase and serum calcium levels in tibial fracture healing process of *Rattus norvegicus*. This study suggest that therapeutic ultrasound (in doses differing from the usual dose) can accelerate bone healing process.

REFERENCES

1. Sousa CP, Dias IR, Lopez-Peña M, Camassa JA, Lourenço PJ, Judas FM, et al. Bone turnover markers for early detection of fracture healing disturbance: A review of the scientific literature. Ann Braz Acad Sci. 2015;87(2):1049-61. doi: 10.1590/0001-3765201520150008.
2. Fontes-Pereira AJ, Amorim M, Catelani F, Matusin DP, Rosa P, Guimarães DM, et al. The influence of low-intensity physiotherapeutic ultrasound on the initial stage of bone healing in rats: An experimental and simulation study. J Therapeut Ultrasound. 2016;4:24.
3. Fontes-Pereira AJ, Teixeira RC, Oliveira AJB, Pontes RWF, Barros RSM, Negrao JNC. The effect of low-intensity therapeutic ultrasound in induced fracture of rat tibiae. Acta Ortop Bras. 2013;21:18-22.
4. Ajai S, Sabir A, Mahdi AA, Srivastava RN. Evaluation of serum alkaline phosphatase as a biomarker of healing process progression of simple diaphyseal fractures in adult patients. Internat Res J Biol Sci. 2013;2(2):40-3.
5. Warden S. A new direction for ultrasound therapy in sports medicine. Sports Med. 2003;33(2):95-107.
6. Warden SJ, Fuchs RK, Kessler CK, Avin KG, Cardinal RE, Stewart RL. Ultrasound produced by a conventional therapeutic ultrasound unit accelerates fracture repair. Physical Ther. 2006;86:1118-27.
7. Michlovitz S, Sparrow K. Therapeutic ultrasound. In: Michlovitz S, Bellew J, Nolan T, eds. Modalities for therapeutic intervention. 5th ed. Philadelphia: F.A. Davis Co; 2005. p. 85-106.
8. Basford JR, Baxter GD. Therapeutic physical agent. In: Frontera WR, ed. Delisa's physical medicine and rehabilitation. 5th ed. Philadelphia: Lippincott Williams and Wilkins; 2010. p. 1696-8.
9. Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. J Orthopaed Res. 1984;2:97-101.
10. Rozen N, Rubin G, Alexander L, Dina L. Development of a new device implementing standardized closed fracture in the rat. Am J Biomed Engineer. 2012;2(6):293-6.
11. Hoppenfeld S, Murthy VL. Treatment and rehabilitation of fractures. Philadelphia: Lippincott Williams and Wilkins; 2000.
12. Dradjat RS. Pengaruh gelombang ultrasound intensitas rendah terhadap percepatan fungsionalisasi osteoblas [Disertasi]. Surabaya: Program Pascasarjana Universitas Airlangga; 2002.
13. Hantoko S, Dradjat RS. The benefit of low intensity ultrasonic wave to fasten callus formation on tibial fractures. Malang: Majalah Kedokteran Unibrawijaya; 2003 .p. 19(2).
14. Rubin C, Bolander M, Ryaby JP, Hadjiargyrou M. Current concepts review: The use of low-intensity ultrasound to accelerate the healing of fractures. J Bone Joint Surg. 2001;83A(2):259-60.
15. Scott A, Khan KM, Duoniol V, Hart DA. Mechanotransduction in human bone: In vitro cellular physiology that underpins bone changes with exercise. Sports Med. 2008;38(2):139-60.
16. Ulstrup AK. Review article: Biomechanical concepts of fracture healing in weight-bearing long bones. Acta Orthopaedica Belgica. 2008;74:291-302.
17. Komnenou A, Karayannopoulou M, Polizopoulou ZS, Constantinidis TC, Dessiris A. Correlation of serum alkaline phosphatase activity with the healing process of long bone fractures in dogs. Vet Clin Pathol. 2005;34:35-8.
18. Cameron M. Ultrasound. In: Physical agents in rehabilitation from research to practice. Missouri: Saunders; 2003. p. 185-218.
19. Draper D, Prentice W. Therapeutic ultrasound. In: Prentice WE, ed. Therapeutic modalities for sports medicine and athletic training. 6th ed. New York: McGraw-Hill; 2009. p. 206-53.