Research article

Blue-light emitting diode exposure effect on alkaline phosphatase levels post-orthodontic stabilization in rats

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ABSTRACT

Introduction and Aim: Exposure to blue light-emitting diode (blue-LED) during the stabilization period can prevent relapse by increasing the tension side osteoblast, which is characterized by increased levels of alkaline phosphatase (ALP) in the gingival crevicular fluid (GCF). Increasing ALP levels occurred from day 7 with the highest peak on day 14 post-stabilization. The purpose of this research was to analyze the effect of blue-LED exposure during the stabilization period on ALP levels in the tension side GCF of Wistar rats (*Rattus norvegicus*) at days 0, 3, 7, and 14 post-stabilizations.

Materials and Methods: Ten male Wistar rats aged 2.5-3 months, weighing 200-250 grams were divided into two groups (control and LED group). An orthodontic force of 35 grams was applied to the mandibular inter incisors of Wistar rats using an open coil spring. Blue-LED (490 nm wavelength, 1000 mW/cm² light intensity) exposure for 30 seconds once a day during a 7-day stabilization period. Gingival crevicular fluid was taken with paper points on days 0, 3, 7, and 14 post-stabilizations for ALP levels measurement using an ELISA method. Two-way ANOVA and Post Hoc LSD statistical tests were performed.

Results: The results showed LED group ALP levels were higher than the control group. There has been a significant increase in ALP levels on days 7 and 14 in the control and LED groups.

Conclusion: It was concluded that blue-LED exposure increased tension side ALP levels. Increasing ALP levels occurred from day 7 with the highest peak level on day 14. Blue-LED exposure in the stabilization period is a favorable therapeutic option to accelerate alveolar bone formation due to being non-invasive, easy to apply clinically, and low cost.

Keywords: blue-LED; orthodontic relapse; alkaline phosphatase (ALP).

INTRODUCTION

Tooth movement caused by orthodontic treatment can be potentially unstable and tend to return to the condition before treatment or called relapse (1). The alveolar bone remodelling process is one of determining the orthodontic treatment successful (2). The remodelling process of alveolar bone is a long time needed, and during this process can occur an orthodontic relapse (1). The relapse movement originating from the stretch of the gingival fibers and the periodontal tissue continues until the alveolar bone formation process is completed (4).

Relapse can occur post-stabilization in the opposite direction of orthodontic tooth movement. The osteoblast and osteoclast activity during the relapse process is like the process of remodelling bone during the orthodontic tooth movement period. The osteoblasts and osteoclasts activity that occurs in the orthodontic relapse process was indicated by increasing osteoclasts in the compression area and decreasing osteoblasts in the tension area (2). Relapse occurs on days 0, 3, 7, and 14 post-stabilizations, with the osteoblast and osteoclast activity like on days 0, 3, 7, and 14 orthodontic tooth movement periods. The difference is that the tension side during the orthodontic tooth movement period is the compression side during relapse and vice versa. Measurement of ALP levels on days 0, 3, 7, and 14 post-stabilizations to identify biological changes that occur during the initial phase until the beginning of the post-lag phase.

Orthodontic relapse prevention can be done mechanically using a retainer as well as additional pharmacological therapy using drugs or Low-Level Laser (LLL) exposure (5, 6). Low-Level Laser (LLL) and Light Emitting Diode (LED) are often utilized as photobiomodulation therapy (7). Light Emitting Diode is a small device that radiates electromagnetic radiation with low intensity, in units of milliwatts (8). Light Emitting Diode is widely used as an adjunct therapy in orthodontic treatment on subjects either in vitro, in vivo, or human clinical studies (9).

Photobiomodulation is the recent therapeutic approach, non-invasive and relatively low cost, and has been shown to produce beneficial effects, namely increasing tissue regeneration and growth (7,10). Photobiomodulation therapy is often associated with accelerating the proliferation and activity of osteoblasts as indicated by the expression of ALP both in vitro and in vivo (11). Photobiomodulation has a

stimulating effect on bone regeneration that accelerates remodeling of the bone and tooth movement (12). During the alveolar bone remodeling process, photobiomodulation therapy causes orthodontic tooth movement faster through the mechanism of high availability of Adenosine Triphosphate (ATP), thereby helping cell turnover more efficiently (13).

The use of LLL light during the stabilization period with 830 nm wavelength for 17 seconds can reduce orthodontic relapse caused by the increased bone formation in tension areas and decreased osteoclast activity in pressure areas (5). Photobiomodulation therapy increases the proliferation of osteoblastic-like cells and ALP (14). ALP is a biomarker of bone formation that decreases in the tension side during orthodontic relapse. Therefore, manipulation of bone remodeling to increase ALP levels is a new strategy to accelerate bone formation with the result that prevents relapse (6).

Light Emitting Diode with 618 nm wavelength, 20 mW/cm^2 intensity, and an exposure time of 20 minutes once a day for 10 days in the active period of orthodontics on 20 Wistar rats, showed an increase in orthodontic tooth movement in the treatment group (15). ALP can be collected in the GCF on the tension side during orthodontic treatment, this indicates the presence of osteoblast activity (2). Measurement of ALP levels in GCF on days 0, 3, and 7 of orthodontic tooth movement with blue-LED exposure on guinea pig incisors, showed an increase in ALP levels from day 3 and the highest ALP levels occurred on day 7 (16). Measurement of ALP levels on days 0, 3, 7, 14, and 21 post-stabilizations by administering intrasulcular injections of carbonated hydroxyapatite (CHA) and carbonated hydroxyapatite combined with advanced platelet-rich fibrin (CHA-aPRF) during the stabilization period, showed an increase in ALP levels occurred from day 7 with the highest level on day 14 and began to decrease on day 21 post-stabilization (6).

In this research, Wistar rats (*Rattus norvegicus*) were exposed to the blue-LED 490 nm wavelength and 1000 mW/cm^2 intensity for 30 seconds during the stabilization period to study the ALP levels on the tension side of GCF at days 0, 3, 7, and 14 after orthodontic stabilization.

MATERIALS AND METHODS

Animal and groups preparation

The Health and Medical Research Ethics Committee of the Faculty of Dentistry, Universitas Gadjah Mada has already approved this research. This study used 10 male Wistar rats, 2.5-3 months old and measuring 200-250 grams, which was conducted at the Integrated Research and Testing Laboratory (LPPT) Unit IV Universitas Gadjah Mada. Ten Wistar rats that will be used as experimental animals were acclimatized in individual cages for 1 week. Wistar rats were chosen randomly and split into 2 groups, namely control groups (without blue-LED exposure) and LED groups (with blue-LED exposure).

Orthodontic appliance preparation and installation

Edgewise standard bracket is cut vertically into 2 symmetrical parts, each bracket is welded on a matrix band (dimension 1 cm × 0.4 centimeters) which is shaped like a ring. Ketamine (35 mg/kg body weight) and Xylazine (5 mg/kg body weight) intramuscularly in the upper right back thigh for general anesthesia was performed before the matrix band was placed on both mandibular incisors of Wistar rats. The labial and lingual surfaces of the right and left mandibular incisors were cleaned with pumice.

GC Fuji I application on the inside of the matrix band that had been welded with a bracket, then the matrix band was installed at 3 mm from the incisal of both mandibular incisors of Wistar rats. Installation of 0.016" round stainless-steel wire and 0.010" x 0.030" nickel titanium open coil spring in the bracket slot which is ligated with the black color of power O. Compression is carried out for open coil spring length (25%) to produce a force of 35 grams which is using a tension gauge to measure. The stainless-steel wire length is 4 mm longer than the open coil length as compensation for the occurrence of tooth movement.

The orthodontic force was applied for 7 days, then stabilized by placing a stopper on the distal right and left mandibular incisor bracket using GC Fuji I for 7 days. Before the stopper is installed, it is ensured that the open coil spring is in a passive condition by measuring the length of the open coil spring which is the same length as the inter-bracket distance, and measuring using a tension gauge shows a force of 0 g.

Blue-light emitting diode exposure

Ketamine (35 mg/kg body weight) and Xylazine (5 mg/kg body weight) intramuscularly in the upper right back thigh for general anesthesia is always performed before blue-LED exposure. The Light Cured LED used is the Light Cured LED D Guilin Woodpecker Medical Instrument, China (490 nm wavelength and 1000 mW/cm^2 light intensity). Blue-LED exposure was given to the gingival center of resistance area of the mandibular incisor roots (7 mm below the cervical tooth) from the labial direction with a tip and gingival distance of 5 mm and an exposure angle of 90°. A light-cured LED is placed on the microphone stand to maintain an angle of 90° during exposure. Blue-LED exposure was carried out for 30 seconds once a day, every 10.00 am for 7 days during the stabilization period in the LED group (Fig.1).

Removal of orthodontic appliances in all groups was performed after 7 days of stabilization. Ketamine (35 mg/kg body weight) and Xylazine (5 mg/kg body weight) intramuscularly in the upper right back thigh

for general anesthesia was performed on Wistar rats before removing of the orthodontic appliance.

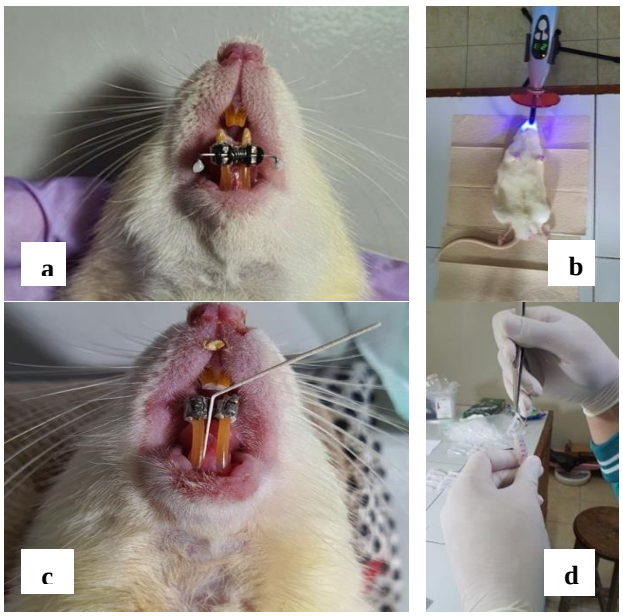

Fig.1: a. Orthodontic appliance has been installed in the mandibular incisors of Wistar rats; b. B*lue*-LED exposure; c. Collecting GCF sample using a *paper point* on day-0, 3, 7, dan 14 post-stabilization; d. Storage of paper points after collecting GCF sample into Eppendorf tubes containing PBS solution.

Gingival crevicular fluid collection and measurement of alkaline phosphatase levels

Ketamine (35 mg/kg body weight) and Xylazine (5 mg/kg body weight) intramuscularly in the upper right back thigh for general anesthesia was always performed on Wistar rats before collecting GCF. Collect GCF of the right mandibular incisor (mesial) for ten Wistar rats in the control group and LED group on day 0 immediately after removing the orthodontic appliance, then on day 3, day 7, and day 14 every hour at 10.00 am.

Samples were collected with methylcellulose paper points placed in the sulcus or at the site of the beginning of the sulcus (17). Collecting GCF in the gingival sulcus can be done using 3 paper points of size 15 with a depth of 1 mm alternately each for 30 seconds with 90 seconds intervals to maximize the volume of the GCF sample (16). Paper points were put into an Eppendorf tube of 350 µl Phosphate Buffer Saline (PBS) and then labeled according to the group name (K-0, K-3, K-7, K-14, LED- 0, LED-3, LED-7, and LED-14). Samples were kept in storage at -80°C until the ALP level test was carried out.

The sample must be at room temperature at the time of the test procedure. The Eppendorf tube of samples was 2000 rpm centrifuged at room temperature for 20 minutes. A sample of 40 L was put into a microplate well, then 10 L of anti-ALP antibody was added to each well and 50 L of streptavidin-HRP was added to each well. The plate was closed with a sealer and incubated at 37°C for 60 minutes. The sealer was opened, and the plate was rinsed 5 times with a wash buffer. A total of 50 L of substrate solution A, then 50 L of substrate solution B was added to each well. The plate was closed with a sealer and incubated at 37°C for 10 minutes. As much as 50 L of stop solution was added to each well, then the blue color will change to yellow. After the stop solution was added, the optical density (OD value) of each well was measured using a microplate reader set at 450 nm wavelength for 10 minutes. The result in the form of an OD value is then calculated into the concentration (level) of ALP in units of units per milliliter (U/ml).

Statistical analysis

Two-way ANOVA was carried out to determine differences between groups and interactions between groups, followed by the Post Hoc LSD test to find out which groups had differences. Statistically significant was shown with a p-value of 0.05.

RESULTS

The results are shown in Table 1 and Fig. 2 that the ALP levels in the blue-LED exposure group were greater than in the control group.

Table 1: The mean value and standard deviation of ALP levels (U/ml) on the tension side GCF after orthodontic stabilization of Wistar rat (*Rattus novergicus*) teeth

Group	Mean ± Standard Deviation			
	Day-0	Day-3	Day-7	Day-14
Control group	0.104±0.003	0.106±0.003	0.166±0.003	0.245±0.003
LED group	0.158±0.002	0.161±0.002	0.494±0.003	0.985±0.003

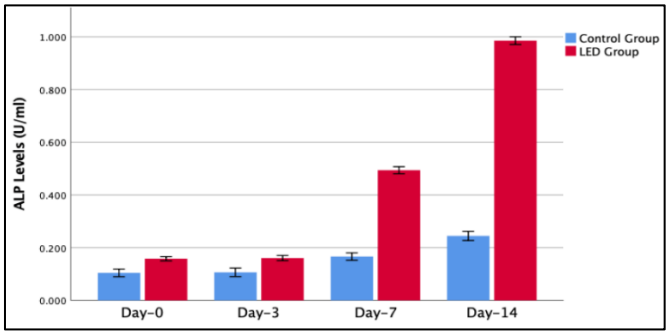

Fig. 2: Mean (Standard deviation) of ALP levels (U/ml) from 2 groups tested

ALP levels in the control group and the LED group increased from days 0, 3, 7, and day 14. Changes in ALP levels in the LED group from day 0 until day 3 showed a slight increase, from day 3 until day 7 seemed to increase sharply up to 3 times, and from day 7 until day 14 increased up to 2 times higher than the previous level. Changes in ALP levels in the control group from day 0 until day 3 showed a slight increase, from day 3 until day 7, and from day 7 until day 14 each increased 1.5 times compared to levels previously.

Table 2: Two-way Anova test on ALP levels

Variable	F	p value
Observation day	69590.280	.000
Treatment	115479.669	.000
Observation day * Treatment	35175.892	.000

Table 2 shows significant differences in ALP levels between the groups during observation days 0, 3, 7, and 14 ($p<0.05$); there was a significant difference in ALP levels between the treatment group without light exposure (control) and the treatment group with blue-LED light exposure ($p<0.05$); and there is an interaction between the day of observation and treatment ($p<0.05$).

Table 3: Post Hoc LSD test differences between groups on the day of observation

Group	Day-0	Day-3	Day-7	Day-14
Day-0	-	.069	.000*	.000*
Day-3	.069	-	.000*	.000*
Day-7	.000*	.000*	-	.000*
Day-14	.000*	.000*	.000*	-

Significant difference ($p<0.05$).

Table 3 shows that there is a significant difference in ALP levels ($p<0.05$) between all groups on the day of observation, except on the day-0 and day-3 days of observation ($p>0.05$).

DISCUSSION

This study used blue-LED light with 490 nm wavelength and 1000 mW/cm^2 intensity, 30 seconds exposure time was given once a day for 7 days stabilization period on the mesial side mandibular incisors of Wistar rats. The sample in this study was taken on the mesial side to determine osteoblasts activity during bone formation on that side.

This study showed that the LED group had greater ALP levels than the control group (Table 1). The administration of LLL as photobiomodulation therapy can inhibit relapse as indicated by increasing bone formation on the tension side (5). Penetration of LED exposure into periodontal ligament cells such as osteoblast in the LED group caused increasing ALP levels. Osteoblasts have a function in the bone apposition process. Osteoblasts produce ALP so increasing osteoblasts due to blue-LED exposure can be indicated by increasing ALP levels. Photobiomodulation therapy can accelerate the proliferation and differentiation of osteoblasts for bone formation as indicated by the expression of ALP (11). Research on blue-LED exposure 490 nm wavelength and 1000 mW/cm^2 intensity during the active period of orthodontics showed the results of increasing ALP levels higher than the control group (16).

Photobiomodulation therapy is usually given in several sessions, which is between 1-10 times of therapy with intervals between 2 times a week to 2 times a day, and the exposure time is usually around 30-150 seconds (18). The optimal exposure time of blue-LED with 490 nm wavelength and 1000 mW/cm^2 intensity is for 30 seconds (15). Photochemical reactions can modulate the biological processes triggered by photons with wavelengths shorter than 600 nm or longer than 1100 nm (18). Blue-LEDs with 400-514 nm wavelengths can penetrate the tissue with a depth range of 0.5-2 mm (19).

Osteoblasts play a major role in bone formation. The function of osteoblasts is to inhibit osteoclast activity by secreting OPG which can prevent RANKL from binding to RANK and secrete ALP which plays a role in bone formation. Osteoblasts control mineralization by regulating calcium and phosphate ion pathways across the surface of the osteoblast membrane. Osteoblasts form bone through two stages, starting with the extracellular matrix formation and then the next stage is the mineral crystals deposition in the matrix. The osteoblast differentiation regulation is operated by the release of transcription factors. Photobiomodulation exposure can also induce changes in transcription factors by influencing the redox reactions of cells (20).

Increasing osteoblast activity during bone formation followed by increasing ALP level in GCF. The activity of the ALP enzyme is considered an indicator of the presence of active osteoblast cells from osteoprogenitors, as well as new bone formation activity (21). ALP is a biomarker of bone formation that is reduced on the tension site during the relapse process that can occur after orthodontic stabilization, in which case manipulation of bone remodeling to increase ALP levels is a new strategy to accelerate bone formation to prevent relapse (6). ALP which is expressed by pre-osteoblasts will be needed locally for deposition and new bone matrix mineralization, so ALP plays an important role in the osteoblastogenesis process. The function of ALP in the mineralization process is to hydrolyze pyrophosphate and provide inorganic phosphate to promote mineralization (22). The ALP enzymes prepare an alkaline atmosphere within the formed osteoid tissue, facilitating calcium deposition within the tissue (23).

Osteoblast and osteoclast activity during the relapse process is like the bone remodeling process due to orthodontic tooth movement, showing an increase in the number of osteoclasts in the compression area and an increase in the number of osteoblasts in the tension area. Relapse occurs in the opposite direction to orthodontic tooth movement. On days 0, 3, 7, and 14 post-stabilizations occur a relapse with the same osteoblast and osteoclast activity on days 0, 3, 7, and 14 of the orthodontic tooth movement periods. The difference is that the tension side during the orthodontic tooth movement process is the compression side during relapse and vice versa. Measurement of ALP levels on days 0, 3, 7, and 14 post-stabilizations to identify biological changes that occur during the initial phase to the beginning of the post-lag phase in the orthodontic tooth movement period. Measurements of ALP levels on day 0 (immediately after removal of orthodontic appliances) and day 3 post-stabilization were carried out to determine the enzymatic changes in ALP due to a very rapid relapse at that time. Measurement of ALP levels on day 7 after stabilization was carried out to observe the lag phase which hyalinization tissue, while day 14 was carried out to observe the continuation of the lag phase and the initial of the post-lag phase (4).

The results showed that the ALP levels in the control group and the LED group increased significantly on days 7 and 14 ($p<0.05$) (Table 3). Increasing ALP levels on days 7 and 14 post-stabilization of the LED group and the control group experienced a significant increase, but the increase in the LED group was greater than the control group. ALP levels in the LED group increased significantly on days 7 and 14 post-stabilization, which was 3 times on day 7 and 2 times on day 14 compared to the previous levels. ALP levels in the control group increased significantly on days 7 and 14 post-stabilization, each of which increased 1.5 times compared to the prior levels (Table 1). These results are consistent with the results of the research of intrasulcular injection of CHA and CHA-aPRF during stabilization, that ALP activity showed an increase on days 7 and 14 after orthodontic stabilization (6).

Alveolar bone remodeling begins with osteoclast activity for 3-5 days, then apposition by osteoblasts for 5-7 days, then a lag phase which is a hyalinization phase for 7-14 days (24). Relapse occurs rapidly after orthodontic appliances are removed (25). Orthodontic relapse begins within 2 hours after the orthodontic appliance is removed and lasts for a short time of about 1-4 days with a decreasing speed and then stopping temporarily. The stopped orthodontic relapse process could be due to hyalinization of the periodontal ligament. Impaired blood flow caused by the force of the stretch of the gingival fibers and periodontal tissue that occurs during the alveolar bone formation process will lead to the formation of hyalinized areas so that the relapse movement stops. Hyalinization tissue in the lag phase occurs due to the application of heavy or light orthodontic forces, but hyalinization tissue is more commonly found at heavy force. Recruitment of cells and preparation of the microenvironment for the periodontal ligament and alveolar bone remodeling occurs during the lag phase (4). Increasing ALP levels on days 7 and 14 occurs because osteoblasts are still activated.

There was an increase in osteocyte apoptosis on days 0 and 3 post-stabilization. Increasing osteocyte apoptosis can lead to increased RANKL production which can interact with RANK and triggers pre-osteoclasts differentiation into osteoclasts (26). There was no increase in ALP levels on days 0 and 3 after stabilization caused by the existence of osteoclast activity while the osteoblasts were not yet activated.

Observations in this study were conducted until day 14, so it could not analyze changes in ALP levels after day 14. This study did not observe the compression side, so it could not analyze osteoblasts and osteoclasts activity on that side. This research is at the experimental animal level, so further research is still needed in clinical trials to be able to analyze blue-LED light exposure effects after orthodontic stabilization as photobiomodulation therapy in patients.

CONCLUSION

Blue-LED exposure (490 nm wavelength and 1000 mW/cm² intensity) in the stabilization period increased ALP levels in the GCF of the tension side after orthodontic stabilization of Wistar rat (*Rattus norvegicus*) teeth. Increasing ALP levels in the tension side of GCF in the mandibular incisors of Wistar rats (*Rattus norvegicus*) occurred from day 7 with the highest peak level on day 14 after orthodontic stabilization. Blue-LED exposure in the stabilization period is a favorable therapeutic option to accelerate alveolar bone formation because it is non-invasive, easy to apply clinically, and relatively low cost. This research is at the experimental animal level, so further research is still needed in clinical trials to be able to analyze blue-LED light exposure effects after orthodontic stabilization as photobiomodulation therapy in patients.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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