Histology of Epiphyseal Plate of Adolescent Rat Stimulated by Laserpuncture

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Abstract. Epiphyseal plate was used for determining longitudinal bone growth. Laserpuncture was believed to stimulate height growth. We used 40 male Wistar rats which aged three weeks old and weighed more than 40 g as subjects. They randomly divided into group A or B and each group evenly divided into four subgroups which were a negative control and others applied with laser on GV20, ST 36 or combination of GV 20+ST 36 respectively. These acupoints were then stimulated using the laser. After treatment, mice were sacrificed, then tibias were taken for histology preparation processes. By light microscope, epiphyseal plate (EP) height (µm) and chondrocytes hypertrophy (CH) height were measured at six equidistant points, and the values were averaged to obtain a final result for each section. Collected data were analyzed using ANOVA test, and the significant value was set up p< 0.05. The mean of EC and CH were lower than control, but mean of ratio EP/CH were higher than control. However, ANOVA showed that there did not differ significantly (p=0.36).

1. Introduction

Epiphyseal plate also known as growth plate has been understood as a target organ for longitudinal bone growth which determines a final height. This plate contains organized cartilage structure located between the epiphyseal and metaphyseal bone at the distal ends of the long bones [1,2,3].

Histologically, the epiphyseal plate consists of horizontal zones of chondrocytes at different stages of differentiation. The germinal zone, or resting zone lie toward the epiphysis of the growth plate, contains immature mesenchymal chondrocytes, which are small, round, singly or distributed in pairs within a compact extracellular matrix (ECM). Beneath it is the proliferative zone, chondrocytes...
assume a flattened, discoid shape and form column-like structures undergo longitudinal cell division and direct the lengthening of the bone in one dimension. And then, proliferating chondrocytes lose their capacity to divide; they differentiate and become prehypertrophic, together with a size increase. Proliferating chondrocytes are located in the transition (maturation or prehypertrophic) zone. In the hypertrophic zone, round cells produce matrix proteins in large amounts. This stage is characterized by abundant in intracellular calcium concentration, which is essential in the production of matrix vesicles. \[4,5,6,7,8,9\].

Longitudinal bone growth takes place by a process called endochondral ossification, in which a cartilaginous scaffold is replaced by bone in a coordinated fashion. It is regulated by genetic, hormonal growth, and environment factors. The major systemic hormones that control longitudinal bone growth during childhood are GH, insulin-like growth factor 1 (IGF-1), the thyroid hormones triiodothyronine (T3) and thyroxine (T4), and glucocorticoids. During puberty, the sex steroids play the most significant role. Whereas, locally it regulated by factors such as Indian hedgehog (Ihh), parathyroid hormone–related peptide (PTHrP), and fibroblast growth factors (FGFs) \[3,4,5,8,9\].

Acupuncture has been used to treat various diseases and maintain healthy since thousand years ago. The term acupuncture is derived from the Latin radicals “acus and pungere” meaning needle and puncture. Acupuncture therapy is an act of needles on specific areas of the body to get the desired effect. This area is known as acupuncture points spread across the surface of the body. Selection of points in acupuncture therapy based on the indication and the desired effect. In contemporary western medicine, acupuncture is considered a form of sensory stimulation that affects several classes of afferent nerve fibers and once directed to the spinal cord and the higher centers in the CNS, it triggers the neurophysiological events that generate the therapeutic outcome. The stimuli generated by acupuncture can activate the autonomic centers and Hypothalamic Pituitary- Adrenal (HPA) axis and, thereby, help to maintain homeostasis and improve the efficiency of several systems of the organism \[10,11\]. Needling acupuncture is believed to increase levels of endorphins and levels of GH in the body.

LASER is the acronym of Light Amplification by Stimulated Emission of Radiation. According to the doses, the laser is divided into the hard laser, medium laser, and low-level laser. The low-level laser which applied to specific acupoint to stimulate body system is known as laserpuncture. Laser is an electromagnetic wave which can inhibit and biostimulate biological tissue, such as improving cellular activities, nervous regeneration in central or peripheral, and also taking roles in hormones and enzymes production. Laserpuncture is one modality of low power laser therapy, non-invasive form of phototherapy which is defined as a traditional acupuncture point stimulation with low intensity and non thermal laser irradiation. Noninvasive and painless nature of the laser is useful to provide comfort and relieve the fear of children. Although the use of low-power, laserpuncture has the same principles of traditional acupuncture and capable of the same biological effects with acupuncture needles \[12,13,14,15,16,17\]. If acupoint is stimulated by laser with particular dose, it will effect on the body system. The impulse in the form of laser is delivered by the nervous system to higher center and will stimulate hormonal releasing from hypothalamus, and then stimulate hypophysis, and it eventually will release growth factor which influences growth \[16,17,18\].

Compared to the needle, the laser has more beneficial such as no pain, no termal, precise doses, effective and efficiency. Laserpuncture technique combines the advantages of traditional Chinese acupuncture with modern medicine \[14\].

In this context, the aim of this study was to present histology changes (epiphyseal plate height and chondrocytes hypertrophy height) of epiphyseal plate of adolescent rats stimulated by laserpuncture.

2. Material and methods

2.1. Ethic Statement
Mice kept and bred according to Institutional Guidelines of the Gadjah Mada University Jogjakarta, approved by Laboratorium Farmakologi dan Farmasi Klinik UGM Jogjakarta.
2.2. *Animal Model*

Forty male Wistar rats which aged three weeks old and weighed more than 40 g randomly divided into group A or B. Each group evenly divided into four subgroups which were a negative control and three subgroups that were stimulated by laser on GV20, ST 36 or combination of GV 20+ST 36 respectively. These acupoints were then applied using the KX Laser GX-2000B which emitted cold red laser (635–680nm/5mW). Laserpuncture was performed 60 seconds/day for ten days (group A) and 15 days (group B). [Fig. 1]

![Figure 1. schema design of study](image)

2.3. *Laserpuncture*

Laserpuncture was performed by laser stimulation using KX Laser GX-2000B (Kangxing), a semiconductor-based low-level laser therapy (LLLT) device emitting a cold red laser (635–680nm/5mW). Before being a laser beam, first, determine the point ST 36 and GV 20. The area around the points was shaved gently with a razor, then laser affixed, a perpendicular laser is turned on for 60 seconds. Control rats were operated with the inactivated probe which was turned off. Acupoint ST 36 is located at two lower limbs, approximately 1mm lateral to the tibial tuberosity, whereas GV 20 located at the peak of head. [Fig.2]

![Figure 2. Laserpuncture on GV 20 acupoint](image)
2.4. Histology Analysis
Following cervical dislocation, the tibiae were resected for free from other tissues. The specimens were fixed in 10% saline buffered formalin at room temperature for two days, then decalcified in 10% HNO3 for 72 hours. After this, it was prepared for following histological processing: dehydration, diaphanization, paraffin embedding, and microtomy, longitudinal section was performed to a thickness of 3µm sections. The sections were stained with hematoxylin-eosin (HE).

Histology structure was assessed by looking epiphyseal plate height and chondrocytes hypertrophy height. Measurement was made by using a camera attached to the light microscope and an image program. Epiphyseal plate (EP) height (µm) was measured at six equidistant points, and the values were averaged to obtain a final result for each section (Fig. 3). Chondrocytes hypertrophy (CH) height (µm) was measured at six equidistant points, and the values were averaged to obtain a final result for each section. The hypertrophy zone is limited by the first hypertrophic chondrocyte on the upper side and by the first mixed trabeculae on the lower side (Fig. 4). A ratio of the epiphyseal plate height to chondrocyte hypertrophy height (EP/CH) was calculated to assess the histology changes.[Fig.3 and 4]

2.5. Statistical Analysis
Data were expressed as means with standard deviations (SD). All data were analyzed using SPSS version 18.0 for Windows. Statistical significance was determined using ANOVA. Significance was accepted at P < 0.05.

3. Result

3.1. Histology Analysis
Measurements of epiphyseal plate zone heights were taken in the central part. The start of the proliferative zone was defined as the point at which the round, individual cells of the resting zone start to become disc shaped and organized into columns. The start of the hypertrophic zone was defined as the point at which proliferative zone chondrocytes round up and become larger. The end of the hypertrophic zone was defined as the vascular invasion front.

The means of EP, CH and ratio EP/CH can be seen in table 1.
Table 1. Height epiphyseal plate (EP) \( \mu \text{m} \), chondrocyte hypertrophy (CH) \( \mu \text{m} \), and ratio EP/CH.

|                | Mean EP ± SD | Mean CH±SD | Mean EP/CH±SD |
|----------------|--------------|------------|---------------|
| Control A [A1]| 482.202 ± 79.189 | 323.892 ± 73.385 | 1.550 ± 0.493 |
| GV20 A [A2]   | 418.802 ± 66.297 | 283.402 ± 47.228 | 1.496 ± 0.294 |
| ST36 A [A3]   | 438.812 ± 40.652 | 291.592 ± 66.983 | 1.520 ± 0.262 |
| GV20+ST36 A [A4]| 405.732 ± 35.763 | 274.332 ± 11.587 | 1.474 ± 0.121 |
| Control B [B1]| 419.952 ± 65.452 | 274.462 ± 59.623 | 1.550 ± 0.141 |
| GV20 B [B2]   | 341.362 ± 90.078 | 248.642 ± 54.308 | 1.295 ± 0.215 |
| ST36 B [B3]   | 381.402 ± 74.164 | 219.302 ± 52.985 | 1.692 ± 0.331 |
| GV20+ST36 B [B4]| 385.252 ± 59.365 | 235.352 ± 59.596 | 1.692 ± 0.286 |

![MEAN EP](image1)

![MEAN CH](image2)

![MEAN EP/CH](image3)

Figure 5. Mean epiphyseal plate (EP) height, mean chondrocyte hypertrophy (CH) height, mean ratio epiphyseal plate/chondrocyte hypertrophy (EP/CH). 1. Control, 2. GV20, 3. ST36 and 4. GV20+ST36

3.2. Statistical Analysis.
There are histological changes that can be seen by increasing and decreasing ratio EP/CH depending on a specific acupoint. However, test ANOVA showed that did not differ significantly (mean EP \( p=0.083 \), mean CH \( p=0.123 \), and mean EP/CH \( p=0.55 \), respectively).
4. Discussion
The usage of laserpuncture is widespread with varying indications and varying dosage, but the study on the effect of low-level laser that is used in laserpuncture in epiphyseal plate are few. In this study, we used a low-level laser with 635 nm wavelength, five mW power, and it were given for 60 seconds, so the energy density was 0.3J/cm². By specific acupoint, treatments were performed in different amounts of time, in which group A received the laser ten times in 10 days, while group B gained laser for 15 days. Here, 2 points were used, namely, point GV 20 that is located at the top of the head and point ST 36 that is located in the two lower limbs (Fig.6). Anatomically, the GV20 point located at the top of the head is adjacent to the brain, and it is associated with the frontal lobes. Whereas ST 36 points were used because it stimulate releasing endorphin, ghrelin, [20] and other neurotransmitters.

![Figure 6. Locations of acupoint used in this study A=GV20, B=ST36 [21]](image)

This study indicates (Fig.5) that histological features are seen from an epiphyseal plate and chondrocyte hypertrophy height differed between subgroups. EP height in each laserpuncture subgroup was reduced, all of the subgroups were lower than control. Also, CH height in each laserpuncture subgroup decreased according to a specific acupoint. It could be seen that the lowest consecutive values were the subgroup of GV20+ST36, GV20 and ST36. Then we look at EP/CH ratio differences. It was seen that laserpuncture effect on EP/CH ratio according to acupoint, where the GV20 was lower than control, whereas the ST36 and GV20+ST36 subgroups were higher than control. However, ANOVA showed that there were not differences between subgroup significantly. Let we look at group A and B differences. When viewed from the duration of stimulation, the mean of ratio EP/CP in group A were smaller than group B, but statistically, there were not differences between A and B significantly.

Unlike this study, Yeom et al. [22] applied laser with a cold red laser (635– 680nm/40mW) on ST36 and SP6 acupoints with energy density 1.2J/cm². They observed a significant increase in mice tibia length. They report that increasing were on hypertrophy zones and growth factors like IGF-1 and BMP2.

Another study, Cressoni et al. [23] evaluated the epiphyseal plate of rats using histological and radiographic analysis after they had been submitted to the application of low-level laser therapy with a wavelength of 830 nm, and doses of 5 J/cm² and 15 J/cm². They observed that low-level laser therapy have no change in bone length. Similar to it, de Andrade, A.R et al. [24] concluded that low laser with wavelength 670 nm in doses of 4J/cm², 8J/cm² and 16J/cm² did not cause changes in the zones of epiphyseal cartilage. Compared with this study, they applied the laser on non-acupoint areas, (tibia and mandible only a side), while this study stimulated lasers on acupuncture points.

5. Conclusion
Our results showed that laserpuncture using a 635-nm semiconductor have an effect on histological changes in adolescent rats, but statistically are not significant. The ratio EP/CH on GV 20 is lower than control, whereas the ratio EP/CH in ST 36 and GV20+ST36 are higher than control.
The interesting is that these changes are not significant. This can be explained, that theoretical acupuncture effect to balance the body functions. Maybe, when done under normal conditions, this effect is useful for optimization. It does not get to the extreme point that causes damage to the network. How the laserpuncture can affect the body system is not clearly known, but what can be explained here is that according to the specific points, laserpuncture is saved enough for stimulation body function.

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