ALP (Alkaline Phosphatase) Expression in Simple Fracture Incident in Rat (Rattus Norvegicus) Femur Bone Supplemented by Apis Mellifera Honey

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Abstract
The aim of the experiment was to observe the serum alkaline phosphatase (ALP) expression of Apis mellifera honey supplementation on simple fracture femur bone. Twelve adult male rats were divided into 4 groups. Those groups are control group, which supplemented with aquadest for 1,5 ml and 3 groups were supplemented with 1,5 ml honey solution (1g/Kg BW, 2g/Kg BW, 3g/Kg BW) for 2 weeks. Fracture incident was made at the day before supplementation by osteotomy technique using dental bur in the metaphyseal region of femur reaching inside the bone marrow. At the fifteenth day, serum was obtained by centrifuging the blood than ass analyzer 24i based on IFCC method. The data were analyzed using ANOVA and Tukey with SPSS for windows. The result showed a very significantly different (P<0. 01) between honey groups and control group and there was no significant different (P>0. 05) between honey groups. The indication of raising ALP revealed that honey might impact osteoblast. The result indicates that gradual dosage on simple fracture might not affect seriously as honey was organic matter containing bioactive compound as minor constituent not in the form of highly intensive extract. Thus, Apis mellifera honey might enhance ALP serum concentration in the incident of simple fracture.

Keywords: ALP; Bone; Honey.

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Introduction
The utilizations of natural honey as medicine by societies are in continuation from immemorial time. In fact, evidences have authenticated that raw honey is the most ancient sweetener, and it was noted to have been used throughout the world several million years ago1. Honey has been experimented to have an ability to preserve bone in the animal model for osteoporosis2. Another study revealed the different result in an experiment of honey on bone healing3. That physicochemical and its antioxidant compound have been expected have the main role in bone cell activity4.

The polyphenolic compound was more than 150 substances including phenolic acid, flavonoids, flavonols, catechins, and cinnamic acid derivative5. Flavonoid was the most studied polyphenol in all natural food. The five mechanisms of flavonoid protecting bone were: i) activation of antioxidant, ii) anti-inflammatory agent correlated in bone injury, iii) pro-osteoblastic agent, iv) anti-osteoclastogenesis, v) through osteoimmunological action6. In addition to polyphenol, saponin has an effect on osteoblast activation leading to bone restoration7. The maintenance of normal condition both structure and function of the skeletal system are based on cells of diverse origin communication including osteoclast and osteoblast8. Incontinuity of bone tissue known as bone fracture was phenomena followed by local inflammation response and led to ossification as bone healing. Bone healing is a unique and effective process involving complex and well-orchestrated interactions between cells, cytokines, osteoconductive matrix and a mechanically stable environment with a good blood supply. The most common therapy for fracture was a surgical procedure. To facilitate
bone healing through osteogenesis, surgeon adds bone grafting, and coadjuvant as osteoconductive and osteoinductive to the surgical treatment⁹. In several cases, such as small bone defect where apposition is absent, non-operative procedure was utilized to recover skeletal injury. In this case, other substances which work to restore the bone tissue are none. Some molecular study has revealed that bone formation was indicated by several markers (i.e. ALP, bone sialoprotein, osteocalcin, collagenase-3)¹⁰,¹¹. The existence of saponin in *Apis mellifera* which has character as estrogen-like bioactivities and polyphenol could affect the bone cell activity¹². The consumption of honey is expected to raise the osteoblast marker such as ALP. Thus, the number of natural honey resources can be beneficial to accelerate bone healing, marked by ALP elevation, as novel supportive herbal medicine in fracture management.

**Materials and methods**

This present study has been approved by Animal Care and Use Committee Faculty of Veterinary Medicine Universitas Airlangga with ethical clearance certificate in number 555–KE. Natural honey was collected from Arjuna Mountain Slope, East Java. Bee colony was *Apis mellifera* that surrounded with *Ceiba pentandra* Tree “Kapok Randu” as the main nectar. Twelve male Rats (*Rattus norvegicus*) with strain Wistar rat were divided into 4 groups randomly. The Rat which used has 150-155 gram range of body weight (BW). Feed and water are provided *ad libitum* with feeder and nipple bottle. The Maintenance of rat was raised in the wire cage in the animal laboratory equipped with natural light. Every single rat was adapted in the animal laboratory environment for a week. The groups consist of control group which was gavaged with aquadest 1ml and 3 supplemented honey groups which was gavaged with gradual dosage of honey (first group 1g/Kg BW of honey ad 1ml aquadest; the second group 2g/Kg BW of honey ad 1ml aquadest; the third group 4g/Kg BW of honey ad 1ml aquadest). The treatment was carried out after surgery. The sterile surgical technique was applied after the adaptation period has been completed. Rats were anesthetized using ketamine HCl (50 mg/Kg BW, Kepro B.V., Deventer – Holland) and xylazine HCl (10 mg/Kg BW, DE Adelaar B.V., Venray – Holland) via intramuscular. Once unconscious, the region of surgery was shaved and sterilized using 10% potassium iodide (PT Mahakam Beta Farma, Jakarta – Indonesia) and 70% ethyl alcohol (PT Inti Medicom Retailindo, Surabaya – Indonesia). One cm incision was laterally made above the Dexter femoro-tibial articulation. The growth plate of femur bone was elevated and defect (1, 2 mm diameter and 6, 3 mm deepness) using trephine bur attached to a dental hand piece with a micro motor as osteotomy. Sterile saline irrigated injured region to avoid heat generation during drilling. The muscle tissue and skin were sutured using chromic catgut 3/0 (PT Medicalindo Primary Source, Medan – Indonesia). The rats were maintained for the aftercare surgery and the clinical symptom was observed.

ALP serum was measured by IFCC method¹³ using prestige 24i automatic biochemical analyser. Blood collection was applied after 14-day treatment post-surgery. Blood was obtained in a cardiac puncture technique and centrifuged in 2500 rpm for 15 min. The data were analysed used ANOVA and Tukey with SPSS for windows.

**Results**

The effect of honey on fracture defect only shows the difference without remarkable⁵. In detail the result of this study was based on, ALP serum as follows (table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>X ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>270.00 ± 31.22³</td>
</tr>
<tr>
<td>1ˢᵗ Honey Group</td>
<td>536.67± 107.74⁶</td>
</tr>
<tr>
<td>2ⁿᵈ Honey Group</td>
<td>533.33 ± 55.07⁷</td>
</tr>
<tr>
<td>3ʳᵈ Honey Group</td>
<td>478.33 ± 76.37⁸</td>
</tr>
</tbody>
</table>

The different superscript alphabet in the same column indicate very significantly different (P<0.01)⁴

**Table 1. Average of ALP Serum**

The average ± SD of ALP serum concentration in control group is 270 ± 31.22 lower level than Honey Group. The average of ALP serum concentration Honey Group is above 400 IU/L. The normal ALP mean of male Wistar rat was 238 IU/L in 149-328 IU/L interval¹⁴.
Discussion

In the fracture condition, the bone defect occurs. Thus, it induced the ossification that existed among localized injury involving the osteoblast and chondrocyte. Fracture healing involved anabolic phase related to de novo recruitment and proliferation of stem cells coming from skeletal and vascular tissues then, followed to a prolonged phase which was predominance by catabolic activity\(^5\). Some factors including fracture healing process were recruitment, proliferation, and differentiation of progenitor cell and revascularization of callus\(^6\).

The interruption of vascular endothelium facilitating inflammation response related to cytokine release. Transforming Growth Factor-\(\beta\)\(^1\) (TGF-\(\beta\)) is chemotactic stimulator which enhances osteoblast precursor and chondrocyte proliferation and may participate in bone cell recruitment in trauma area\(^\circ\). In osteoporotic fracture healing, TGF-\(\beta\)\(^1\) has been known to play role in all bone healing process\(^7\). TGF-\(\beta\)\(^1\) induces bone extracellular matrix production (e.g.: osteopontin, ALP, collagen).

Alkaline phosphatase is a membrane-bound enzyme which exists in almost all living organism. Tissue-nonspecific alkaline phosphatase was dominantly presented in skeleton development. It was caused by the involvement of ALP in mineralization. The activity of ossification is associated with chondrocyte and osteoblast\(^8\).

This experiment study revealed a positive effect of Apis mellifera honey on fracture healing indicated by ALP serum elevation. Normal fracture healing generate and enhance osteoblast activity. Osteoblast accounts for tissue bone matrix, mineralization, and ALP secretion. It was in line that peak enhancement of ALP existed at day 10\(^\circ\) after fracture at dog patient\(^9\). The lowest ALP serum concentration was showed at control group. This owing to the group was in normal fracture condition that makes the ALP elevation was not supported by any compound.

Normal ALP serum interval was 149-328 IU/L\(^{10}\). The ALP serum value of control group, 238 IU/L, was in normal range. It was indicated on the day 14\(^\circ\), the osteoblast activity was decreased and ALP production fell to normal range. Similar finding to\(^{11}\), that no distinctive histological differences in untreated fracture at day 14\(^\circ\) including the formation of hyaline cartilage and ossification were observed. It could be a notion that the fracture healing got into a catabolic stage.

Honey supplementation in this experiment showed very significantly different (\(P = 0.006\)). It was in line with\(^{12,13,14}\), that indicated efficacy of honey could prevent bone loss. The phytochemical compound in honey produced by Apis mellifera was Alkaloid, Flavonoid, Saponin, and Glycosides\(^15\). Saponin was estrogen-like bioactivities that could affect osteoblast\(^7\).

That natural product containing phenolic acid, flavonoid and polyphenol has been reported could promote osteoblast-mediated bone formation\(^17\). Honey has flavonoid such as kaempferol, gallic acid, ellagic acid\(^17\), catechin, quercetin, vanillic acid and others\(^18\). Vanillic acid has been reported could exert stimulatory effects in osteoblast-like cells estrogen receptor signaling pathways\(^20\). In vitro study, quercetin and rutin having been reported by\(^21\) could enhance the expression of osteoblast gene marker proliferation of bone marrow mesenchymal stem cell, ALP secretion, and induce mineralization.

Honey might also increase intestinal absorption for calcium by decreased luminal pH. It has been reported that honey could support and produce lactic acid in intestine\(^22\). Bacteria would ferment non-digestible carbohydrate of honey to produce lactic acid that may decrease pH, leading calcium solubility. In vitro study report, non-digestible carbohydrate may indirectly open epithelial tight junction. This may activate paracellular calcium transportation\(^23\). The ability of honey in calcium intake regulation may support mineralization of bone tissue.

The confirmation of osteoblast activity widely exists. Several study reported that honey solution was not significantly increase the bone healing process by histopathology\(^9\). The molecular expression involving bone healing was not examined, such as Runx2 which has role play in osteoblast and chondrocyte differentiation\(^24\) and Transforming Growth factor-\(\beta\)\(^25\). The Alpl gene, which influence the ALP production and bone aging by the mediation of adenosine triphosphate in mesenchymal stem cell (MSCs).

The role of MSCs is osteoprogenitor lineage. Bee venom has been studied on MSCs related to osteocyte differentiation potency\(^26\).
Bee product which has been reported could affect the stem cell. It has been reported that honey mobilizes the stem cell, when the tissue got injured. Saponin, which is contained in *Apis mellifera* honey, significantly increased capillary network forming of rat bone marrow MSCs. It was already known that angiogenesis is a part of fracture healing process.

In this study, it was acceptable that honey may enhance osteoblast activity. However, the comparison between honey groups was not significantly different. It might be the narrow of inter given dosages which lead to do more research with wide range of plausible dosage. The examination of honey in vivo study revealed the ALP expression directly from the animal response involving the injury of the living tissue.

**Conclusions**

In Conclusion, Honey supplementation has a beneficial effect on fracture healing by supporting the osteoblastogenesis. Pro-osteoblastic effect of honey could be revealed by ALP serum increase which indicates proliferation and maturation of osteoblast activity in injured bone tissue. However, the limitation of this study still remained. Further study, related to honey on ALP and its signalling pathway in cell needs to be revealed.

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**Declaration of Interest**

The authors report no conflict of interest.

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