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Ethanolic extract of grape (*Vitis vinifera*) prevents bone defect in the overtraining-induced rat

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ABSTRACT

Introduction: Excessive physical activity can lead to an early aging and degenerative disease such as osteoporosis characterized by decreasing bone density and the number of osteoblasts and increasing the number of osteoclasts. This study aimed to examine the activity of grapes ethanol extract to prevent bone damage in overtraining-induced rats.

Methods: The study design was experimental research using a completely randomized posttest-only control group design. Subjects were 36 male albino rats, aged 2.5 months, weighing 180-200 grams divided randomly into two groups. Control group (n= 18) was treated with overtraining + placebo for three weeks and the treatment group (n= 18) was treated with overtraining + 1.25 g/kgBW grapes ethanol extract for three weeks.

Results: The result showed that the average number of osteoblasts in the control group after treatment was 125.44 ± 7.770 cells per field of view, while in the treatment group was 137.06 ± 12.037 cells per field of view (p<0.01). The control group's bone density was lower than the treatment group (308.84 ± 17.195 vs. 438.11 ± 25.940 µm, p<0.01). In contrast, the number of osteoclasts in the control group after 14 days was higher than the treatment group (19.89 ± 3.411 vs. 13.33 ± 4.485 cells per field of view, p<0.01).

Conclusion: This study suggests that grape extract's antioxidant capacity can prevent a bone defect in the overtraining-induced rat.

Keywords: Grapes, osteoblasts, osteoclasts, bone density, overtraining.
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INTRODUCTION

Physical activity is essential for human life because it maintains and improves health.[1] However, excessive physical activity was found to cause cell damage.[2-5] It could cause bone disorders and premature aging. Excessive physical activity may lead to oxidative stress because it can increase oxygen consumption up to 100-200 times, so it triggers the release of free radicals.[6] Reactive Oxygen Species (ROS) are free radicals that are dangerous to the body. ROS such as superoxide and hydrogen peroxide can cause damage to DNA, proteins, and fats. Free radicals that are produced when excessive physical training occurs are the radical superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·).[7] Generally, 2-5% of the oxygen used in metabolic processes will become superoxide ions so that the current heavy physical activity increased free radical production.[8]

ROS is generated by mitochondria, especially hydroxyl radicals (OH·), that will activate inflammatory pathways through ERK activation and further activate Nuclear Factor-kB (NF-kB). NF-kB will eventually stimulate the production of proinflammatory cytokines, such as TNF-α and IL-6. These cytokines will increase osteoclastogenesis, inhibiting apoptosis of osteoclasts and inhibit the activation of osteoblasts.[9] Epidemiological evidence in humans and animal studies showed a close relationship between oxidative stress and osteoporosis's pathogenesis.[10] Under oxidative stress, osteoclast will proliferate and differentiate.[11] Research indicated that H_2O_2-induced oxidative stress could also inhibit osteoblast differentiation of bone marrow stromal cells through activation of the ERK pathway and ERK-dependent NF- κβ.[12]

Grapes (*Vitis vinifera*) is one of Indonesia's most abundant fruits, a rich source of antioxidants, including polyphenols and anthocyanins.[13,14] Flavonoids are the most potent kind of polyphenol in terms of antioxidants. It could act against ROS by increasing endogenous antioxidant and neutralizes free radicals directly.[15] It can increase endogenous antioxidants by activating Nrf2 by phosphorylating Keap1. Phosphorylated Keap-1 will give a time for newly synthesized Nrf2 to translocate to the nucleus, where it binds to antioxidant-responsive element (ARE), thus induces endogenous antioxidants transcription such as SOD, GSH, and catalase.[16-18] Anthocyanins are also potent antioxidants with high potential as a free radical scavenger and are protective against oxidative stress. Anthocyanins have been proven to prevent lipid peroxidation.[19]

The content of resveratrol in grapes also downregulates TNF-α, thus suppressing IL-6 production.[20] It will lead to osteoclastogenesis inhibition, increasing osteoclast apoptosis and increasing the activation of osteoblasts.[9]

The benefits of grapes are widely recognized empirically by the community. However, scientific evidence is necessary for this plant's benefits in preventing damage to organs in the body, especially bone.

METHODS

A total of 36 3-months-old experimentally naïve male rats, Wistar strain with an average initial body weight of 180-200g, were sampled from animals in Animal Laboratory Unit, Faculty of Medicine, Universitas Udayana. The animals were housed under environmentally controlled conditions (12 hr light/dark cycle; 22–24°C) and provided with food and water *ad libitum*. Animals were allowed to adjust to the new experimental condition for seven days. The ethics committee of animal experiments in the Faculty of Veterinary Medicine, Universitas Udayana, has approved all the protocols involved in animal handling (No. 205/KE-PH-Lit-2-III-2016).

The rats were divided into two groups. The control group (P0) was given an overtraining + placebo, and the treatment group (P1) was given an overtraining + ethanol extract of grape of 1.25 g/kgBW of rat per day. The Overtraining protocol employed was already described in another publication. It was a 60 min swim series until the rats showed a sign of drowning, seven times a week, for three weeks.[2] The swimming apparatus was 80 cm in length, 50 cm in width and 90 cm in depth. The water level was adjusted to 70 ± 5 cm, and the water temperature was maintained at 33–35°C.

Ethanol extract of grape was prepared according to the previously described method.[21] The dried fruit grinded coarsely into a powder. The powder was then extracted with 95% ethanol in the Soxhlet apparatus. The crude extract was then filtered by Whatman paper. The solvent was dried under reduced pressure at a maximum temperature of 50 °C with a vacuum rotary evaporator. The final extract was stored at -20 °C until it was used to feed the rats in the experimental groups.

After 28 days of treatment, rats were sacrificed to collect the bone sample. Necropsy procedures were adapted from Fish *et al.* (2008) method.[22] The femur of each animal was sliced in the size of 1x1x1 cm^3 and immersed in a 10% neutral buffered formalin fixative solution before decalcification with 10% EDTA-2Na solution for three weeks at 4°C. Specimens were then dehydrated through an ascending ethanol series and embedded in paraffin using standard procedures. The procedure of tissue preparation was adapted from Kiernan's method.[23] Hematoxylin-Eosin staining was used in this study. After staining and the mounting process was done, the preparation was ready to be observed with a microscope. Examinations of the bone tissue were done by using both 100x and 400x magnification under a binocular microscope.

Data are presented as group mean ± standard deviation (SD). An independent sample T-test was performed to detect the significant differences between groups. Experimental differences were considered statistically significant if $p < 0.05$.

RESULTS

The results showed the average number of osteoblast in the control group (P0) after overtraining and placebo was 125.44±7.770, whereas in the group treated by overtraining and ethanol extract of grape (P1) was 137.06 ±12.037 (p <0.01). The bone density in the control group (P0) after treatment with overtraining and placebo was 308.84±17.195 µm, whereas in the group treated by overtraining and ethanol extract of grape (P1) was 438.11 ± 25.940 µm (p <0.01). The average number of osteoclast in the control group (P0) after treatment with overtraining and placebo was 19.89±3.411, whereas in the group treated by overtraining and ethanol extract of grape (P1) was 13.33 ± 4.485 (p <0.01) (Fig 1 and 2).

DISCUSSION

The overall study results suggest that grape's ethanolic extract can prevent the occurrence of osteoporosis triggered by

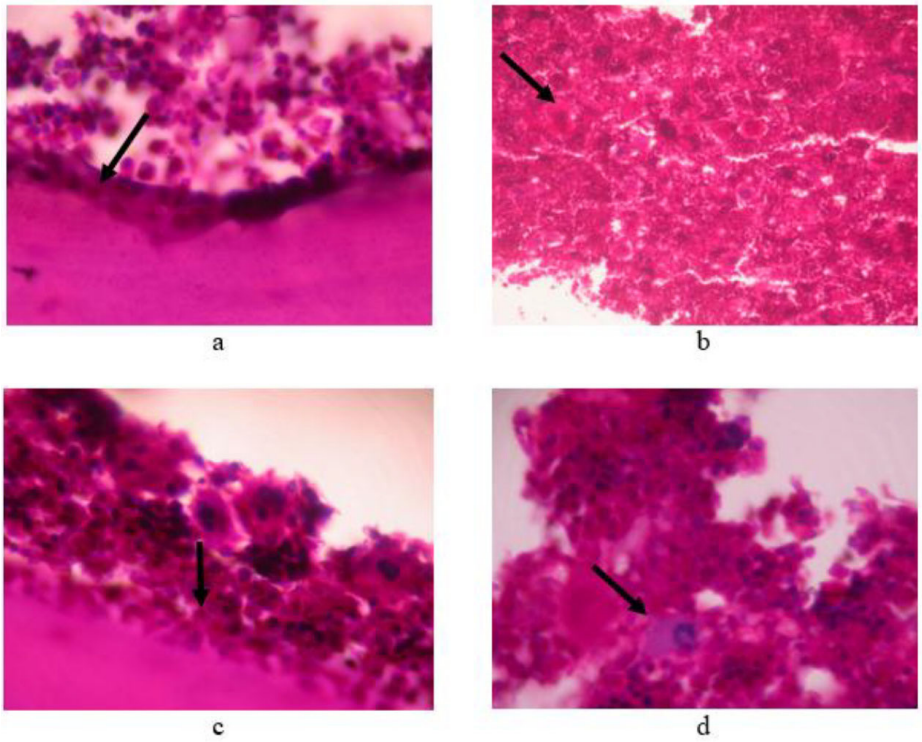
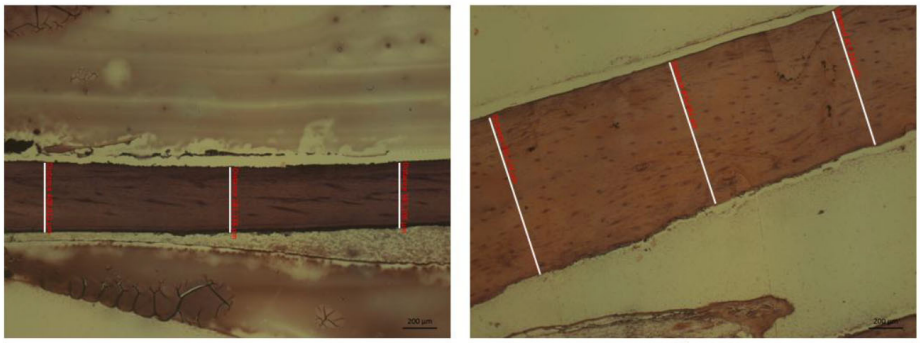


Figure 1. Microscopic observation of bone: (a) & (b) are osteoblast and osteoclast of the control group (P0), respectively. (c) & (d) are osteoblast and osteoclast of treatment group (P1), respectively. (Stained with H&E, 400 times magnification).



a b

Figure 2. Microscopic observation of bone density: (a) control group (P0) and (b) treatment group (P1). (Stained with H&E, 400 times magnification)

excessive physical activity or overtraining through a variety. High level of ROS induces apoptosis of osteoblasts and osteocytes through activation of p66Shc and stimulates transcription factor FoxO which reduces levels of β-catenin. FoxO eventually will inhibit the osteoblastogenesis. Another study showed that oxidative stress induces dephosphorylation of proteins involved in cell cycles such as pRB, P107, and P130 through activation of protein phosphatase 2A (PP2A), which leads to the occurrence of apoptosis in osteoblasts.[24] Oxidative stress has proven to activate the apoptotic cascade and inactivates antiapoptosis lines, such as signaling pathways AKT / mTOR.[25] Other pathways involved in osteoporosis's pathogenesis due to oxidative stress are the extracellular signal-regulated kinase (ERK) and ERK-dependent nuclear factor-kB signaling pathway.[12] Besides JNK pathway-1 also involved in osteoblast differentiation. Additionally, hydrogen peroxide was shown to inhibit transcription of Gli1, Ptch1b, Alp, and BSP, phosphorylates JNK-1 and p38 to inhibit osteoblasts' differentiation.[26]

The ethanol extract of grapes fruit used in this study contains flavonoids much as 183.77 mg/100g QE and total phenol content of 6.25% w/w GAE with the antioxidant activity of 487.98 ppm GAEAC and Inhibition Concentration 50% (IC50%) of 1.72 mg/ml. Ethanol extracts of grapes can prevent oxidative stress on the bones due to polyphenols' high content by directly neutralizing free radicals and increasing endogenous antioxidants. Polyphenols help activate Nrf2 by liberating the Nrf2-Keap1 bond, thus stimulating the transcription of enzymatic antioxidants such as SOD, GSH, and catalase.[16,17] Besides, flavonoids can activate the ERK, JNK, and p38, which were phosphorylated in oxidative stress, further activate Nrf2 resulting in increased gene expression of endogenous antioxidants,[27] and increase the differentiation of osteoblasts.[26] We previously showed that the resveratrol compound has a strong antioxidant effect to reduce oxidative stress.[28,29] This effect makes resveratrol effective enough to protect osteoblasts from oxidative damage and prevent an increase in osteoclasts. Resveratrol was shown to inhibit the oxidative stress through Sirt 1 activation, inhibition of p53 acetylation, activate caspase-9, and Bcl-2, thus inhibits apoptosis and stimulates osteoblast proliferation in MC3T3-E1 cells.[30]

Grapes also contain Quercetin, which also possesses antioxidant activity. Studies showed that Quercetin might protect bone tissue from oxidative damage induced by H2O2 mediated by phosphorylation of extracellular signal-regulated kinase (ERK-1 / ERK 2).[31] Quercetin also reduces the concentration of thiobarbituric acid-reactive and lipid hydroperoxide in the pancreas and increase enzymatic oxidant activity. These results indicate that Quercetin's antioxidative activity may protect bone tissue from damage by reducing oxidative stress caused by various exogenous and endogenous sources.[32] Several kinds of polyphenols like epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), theaflavin (TF) and thearubigins (TR) are also found in grapes.[33] The EGC has been proven can stimulate osteoblast differentiation and inhibit the induction of osteoclast differentiation.[34] EGCG and theaflavin can increase the number of osteoblasts, osteoblastogenesis, and bone formation, increasing osteoblastic survival, proliferation, and bone differentiation.[9] Thus, Grapes contain many natural antioxidants, which have a protective effect against oxidative stress.

CONCLUSION

This study suggests that grape extract's antioxidant capacity can prevent a bone defect in the overtraining-induced rat.

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AUTHORS CONTRIBUTION

All authors declared that they had contributed equally in all research phases, including preparation, experiment, data gathering, analysis, drafting, and approval to publish this manuscript.

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CONFLICT OF INTERESTS

The authors declare that there are no competing interests.

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