Astaxanthin increase osteocytes, osteoblasts, decrease adipocytes cells, and reduces osteonecrotic events in femoral head of Wistar rats exposed to alcohol

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ABSTRACT

Background: Osteonecrosis is the death of bone cells that can be associated with alcohol abuse. Alcohol abuse has been projected to increase by 0.6% every year. The high morbidity and cost of therapy of osteonecrosis necessitate the effort to prevent osteonecrosis before it manifested. Astaxanthin is an antioxidant that is expected to increase the number of osteocyte and osteoblast cells, decrease the number of adipocyte cells, and reduce the incidence of osteonecrosis.

Methods: This is an experimental randomized post-test control only group design. A total of 24 Wistar rats who met the inclusion criteria were randomly assigned to the control group with the administration of alcohol and the treatment group with the administration of alcohol and astaxanthin. The number of osteocytes, osteoblasts, adipocytes, and osteonecrosis occurrence was examined in the first, second, and third week.

Results: Statistical analysis showed a lower average number of osteoblast cells in the control group (38.1.43) than in the treatment group (55.63±2.17) with astaxanthin administration. The same results can also be seen in the number of osteocyte cells in the control group (16.69±0.94) as compared with the treatment group (28.06±1.26). Meanwhile, the average number of adipocyte cells in the control group showed a higher yield (58.69±1.18) when compared with the treatment group (27.50±1.24). The occurrence of osteonecrosis, as depicted by the presence of empty lacunae and necrotic osteocyte cells, showed the highest increase in osteonecrosis occurrence at week 3 (34.56±0.31) of the control group. While in the treatment group, the decrease in the incidence of osteonecrosis showed the lowest number in week 3 (4.75±0.79). The statistical analysis test using one-way ANOVA showed that the mean difference between treatment and control groups was statistically significant (p<0.05).

Conclusions: The above calculation showed that administration of astaxanthin could decrease the incidence of osteonecrosis in the femoral head of rats exposed to alcohol.

Keywords: Osteonecrosis, caput femoris of rat, astaxanthin, osteoblast, osteocyte, adipocyte, alcohol

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INTRODUCTION

Osteonecrosis is the death of bone tissue because of lack of nutrition supplied by blood circulation. It may be caused by both trauma and nontraumatic processes. Prevention is important because osteonecrosis has high morbidity rate that causes a formidable financial burden.

Nontraumatic causes of osteonecrosis are Perthes disease, Caisson disease, Gaucher disease, alcohol consumption, and systemic lupus erythematosus. Osteonecrosis mostly occurs on the bone with single vascularization and limited collateral, such as the femoral head (caput femoris), humerus, carpal, and talus. One-third of nontraumatic cases are related to corticosteroid and alcohol. Studies showed that 10%-74% patients with nontraumatic osteonecrosis consume alcohol. Another study suggested that alcoholism increased the risk of femoral head osteonecrosis. Human and animal studies showed that coagulation disorder and oxidative stress are factors related to osteonecrosis. Vascular blockage is caused by thromboembolism on the blood vessel that nourishes the bone. Estrogen therapy in Turner Syndrome was found to increase the risk of thromboembolism in femoral head. Alcoholism also significantly decrease bone marrow activity in patients with osteonecrosis. Alcohol consumption per capita in Southeast Asia increased during 2005-2010. Indonesia ranked fifth with 0.6% of projected 5-years-increase. From 2000 to 2010, 1153 decompression and hip replacement procedures were performed in Taiwan, 45.2% of which were caused by alcohol-related femoral head osteonecrosis. A 2004 survey of 1502 patients with idiopathic femoral head osteonecrosis, 31% were related to alcohol abuse.
An animal study showed that alcohol exposure increases the size and proliferation of adipocyte tissue in subchondral of femoral head, while also increases bone pressure in the proximal femur. Increase in adipocyte volume is hypothesized as the cause of sinusoid collapse that reduces perfusion in femoral head, leading to osteonecrosis. Clinical deterioration may be related to four interacting factors. They are increased adipogenesis related to osteoprogenitor differentiation, reduced angiogenesis, suppressed expression and proliferation of osteogenic genes in bone marrow, and hypercoagulable state induced by genetic anomalies or other diseases. Weekly alcohol consumption of more than 400 ml clearly increases the risk of osteonecrosis.

Alcohol can induce adipogenesis both in vivo and in vitro. Reduced osteogenic properties and increased adipogenesis were detected in cells that were exposed to ethanol. This contributes to the mechanism of osteonecrosis. Alcohol downregulates PPAR-γ (Peroxisome proliferator-activated receptors), a group of nuclear receptor protein that regulates gene expression and has an important role in the regulation of cellular differentiation and metabolism of carbohydrate, lipid, and protein. There is increase of lipid transport and storage because of the synthesis of aP2 (adipocyte P2) and LPL (lipoprotein lipase) related to alcohol-induced changes in synthesis and expression of PPAR genes that can be measured by Nile red staining in mesenchymal cells. Another study showed that alcohol consumption increased cortisol secretion that contributed to redistribution of adipose tissue, oxidative stress, and activation of CYP2E1 that caused apocrine dysregulation.

Osteonecrosis induced by intragastric alcohol in the mouse model has not been able to show the contribution of proinflammatory response through the toll-like receptor 4 (TLR-4) receptor. Reduced adiponectin in alcohol group was consistent with the hypothesis that femoral head osteonecrosis is caused by ischemia in blood vessels of the femoral head.

Oxidative stress is the pathogenesis of metabolic syndrome and is also strongly related to obesity. Oxidative stress in adipose tissue influences adipokine secretion that reduces the concentration of adiponectin and increases blood free fatty acids. Increased inducible nitric oxide synthase (iNOS) expression in the study showed that osteocyte death as the alternative pathophysiology of alcohol-induced osteonecrosis.

Nitric oxide (NO) is an omnipresent intracellular messenger that modulates blood flow, thrombosis, and neural activity. NO stimulates nearby tissues and interacts with ROS, producing peroxynitrite. Cytotoxicity of peroxynitrite is mediated by the effect of lipid peroxidase, protein nitration, and oxidation. Mitochondrial enzymes are susceptible to peroxynitrite that can reduce adenosine triphosphate (ATP) formation, trigger mitochondrial permeability transition, and open the pores of the cell membrane. These processes withdraw electron transport, ATP formation, and will cause mitochondrial swelling and increased mitochondrial permeability. This will lead to efflux of proapoptotic molecules such as Apoptosis Inducing Factor (AIF) and Cytochrome C. Effector activation that triggers nucleic DNA fragmentation increases mitochondrial damage because this process will activate poly-ADP-ribose-polymerase (PARP). Activation of this enzyme consumes nicotinamide adenine nucleotide (NAD) while free PAR will travel to mitochondria and increase the mitochondrial efflux (nuclear to mitochondria cross talk). Like reperfusion injury, massive oxidation and nitrosative DNA damage will cause cellular apoptosis.

Vascular Endothelial Growth Factor (VEGF) is a special mitogen for vascular endothelial. It was initially identified as endothelial growth factor from follicle cells in bovine hypophysis by Ferrara and Davis Synth. VEGF-A is a dominant factor in angiogenesis regulation and endothelial growth compared to other isoforms. VEGF also promotes chemotaxis of mesenchymal stem cell (MSC). Vasculogenesis and angiogenesis are triggered by the local and systemic increase of VEGF that allows mobilization of osteogenic substrates, stem cell pericyte, and MSC that can differentiate. Hypoxia is a potent stimulus of growth factor expression especially VEGF during angiogenesis. Reduced oxygen pressure will stimulate vascular endothelial to produce VEGF that will trigger angiogenesis that is essential form tendon healing and tendon graft remodeling.

Some studies of osteonecrosis prevention have been conducted. Pitavastatin has anti lipid effect in adipogenesis of rabbits that were injected with 20 mg/kg of methylprednisolone, decreasing the incidence of osteonecrosis. Oral vitamin E reduces the incidence of corticosteroid-induced osteonecrosis.

In another study, no statistical significance was found related to total cholesterol level, triglyceride, and adipocyte size between model group and hydrogen antioxidant group. This finding suggested that disorder of lipid metabolism will not resolve after two weeks observation with the support of hydrogen antioxidant and there are several osteonecrosis preventive mechanisms besides lipid metabolism disorder. This is explained by the role of antioxidant towards hydrogen and the incidence...
of osteonecrosis. Therefore, further studies are needed to investigate the optimal level of hydrogen antioxidant that can prevent osteonecrosis. The investigation of 0.2% astaxanthin (2g/kg) supplementation for visceral adipose and lipid profile in mouses after 6 weeks showed a reduction in visceral adipocyte, reduction in free fatty acid levels, and increased HDL concentration. Astaxanthin can reduce lipid levels and abnormalities. Inverse relationship was noticed in the structure of the trabecula of the femoral head. Histopathological bone specimen was examined to measure the amount of adipocyte, osteoblast, osteocyte and the incidence of osteonecrosis. The trabecula is taken from anterosuperior part of lateral femoral head.

MATERIAL AND METHODS

The investigation was started in August 2017 and conducted at Pharmacology laboratory, Faculty of Medicine Universitas Udayana, Bali for animal intervention, then at Veterinary Pathology laboratory, Faculty of Veterinary Medicine, Universitas Udayana for histopathology examination.

The design of the study is an experimental randomized post-test control only group using Male Wistar rats. There were four animals in each experimental group with the total of 24 rats. Sampling was done using simple randomization because of the homogenous study population. Male Wistar rats, aged 10-14 weeks with the weight of 200-250 gram were selected, and has never been exposed to corticosteroid.

Twenty four male Wistar rats aged between 10-14 weeks were divided into six groups. The first three groups were 0.5 ml intraoral 40% alcohol was given for 1 week in P0 group, 2 weeks in P1 group, 3 weeks in P2 group. The second three groups were 0.5 ml intraoral 40% alcohol and 0.106 mg/kg/day of astaxanthin were given for 1 week in P3 group, 2 weeks in P4 group, and 3 weeks in P5 group. Alcohol was provided every morning at 09.00 AM local time. The mice were each put in a 30x20 cm cages with environment temperature of 20-25°C. They were given 12-20 gram of pellet containing protein (20%), fat (5%), carbohydrate (45%), fiber (5%), vitamins, and minerals as the daily diet. Water was given ad libitum. Astaxanthin was given every morning at 09.00-10.00 local time. The weight of each rat was measured weekly. In the last day of the first week, rats in P0 and P3 were euthanized with 200 mg/kg of intramuscular ketamine. Both proximal femur underwent histopathological examination while the rest of the body was buried.

Histopathological bone specimen processing

Bone samples from euthanized rats were fixated for 1 weeks using 10% formaldehyde-0.1 M phosphate buffer pH 7.4. Then, the samples were decalcified by 25% formic acid for 10 days followed by neutralization by 0.35 M sodium sulfate for 1 day. The specimen then was processed using tissue processor, put on paraffin block, cut by microtome into 4µm thickness, and finally stained using hematoxylin and eosin. The trabecula of the femoral head was examined in order to measure the amount of adipocyte, osteoblast, osteocyte and the incidence of osteonecrosis. The trabecula is taken from anterosuperior part of lateral femoral head.

Data Analysis

Obtained data were analyzed by descriptive analysis, Normality and Homogeneity test (Normality test using Saphiro Wilks Test and homogeneity test = Levene's Test for Equality of Variance), Normal distribution Inferential analysis with One Way ANOVA with Tamhane's post-hoc analysis for intragroup data and unpaired T-test for intergroup data, and abnormal distribution Inferential analysis with Kruskal-Wallis test with Mann-Whitney post-hoc analysis for intragroup and intergroup data.

RESULTS

Experimental data of femoral head osteonecrosis in rats exposed to alcohol and astaxanthin or without astaxanthin for 7 days, 14 days, and 21 days were processed and analyzed in order to evaluate and answer hypothesis and research purposes. The data consisted of qualitative measurement of osteoblast, osteocyte, adipocyte, and incidence of osteonecrosis in trabecula using 200x and 400x zoom of microscopy field were combined with a digital camera to produce digital images. The animal was assigned to 6 groups using simple random sampling: (a) Control group (P0), was given 0.5 cc intraoral 40% alcohol every day without astaxanthin and evaluated on day 7. (b) Control group (P1), was given 0.5 cc intraoral 40% alcohol every day
without astaxanthin and evaluated on day 14. (c) Control group (P2), was given 0.5 cc intraoral 40% alcohol every day without Astaxanthin and evaluated on day 21. (d) Experimental group (P3), was given 0.5 cc intraoral 40% alcohol and astaxanthin 0.106 mg/kg/day every day and evaluated on day 7. (e) Experimental group (P4), was given 0.5 cc intraoral 40% alcohol and astaxanthin 0.106 mg/kg/day every day and evaluated on day 14. (f) Experimental group (P5), was given 0.5 cc intraoral 40% alcohol and astaxanthin 0.106 mg/kg/day every day and evaluated on day 21.

On the day of evaluation, the animals were terminated in order to take the bone sample from both proximal femur for the measurement of osteoblast, osteocyte, adipocyte, and incidence of osteonecrosis. The quantitative data, obtained from microscopy were examined by a histopathologist.

The results were used as the base data for analysis using normality test, homogeneity test, comparative test, and experimental effect.

Sample analysis
The analysis included descriptive data distribution, number of osteoblast, osteocyte, and adipocyte cells in control and experimental group. The data were analyzed using SPSS for Windows version 23.0.

Descriptive analysis
The aim of descriptive data analysis was to get a clear picture regarding data distribution and standard deviation of each variable.

Table 1 shows that total subjects were 24 that were divided into control and experimental group. The number of subjects in each subgroup was 4 or 16.7%

Table 2 shows the mean difference of each variable in week 1. The amount of osteoblast and osteocyte was lower in control group (38±1.43) compared to experimental group (55.63±2.17). The amount of adipocyte in control group was higher (58.69±1.18) compared to the experimental group (27.50±1.24).

The second and third week measurements showed the reduction of osteoblast and osteocyte in control groups and the increase of osteoblast and osteocyte in the experimental groups.

Adipocyte increase was found in control groups (week 2: 88.75±4.66, 111.63±1.53), while reduction was found in experimental group (week 2: 21.00±1.48, 14.63±0.48).

The incidence of osteonecrosis was indicated by empty lacuna. There was an increasing average of empty lacuna in control group (week 3: 34.56±0.31), while the average was decreasing in the experimental group (week 3: 4.75±0.79).
Inferential analysis

The aim of this analysis is to generalize the result into population. The statistical tests used were One Way ANOVA with Bonferroni post-hoc analysis for homogenous data or Tamhane's post-hoc analysis for inhomogenous data. The tests were interpreted using 95% CI and p < 0.05.

Normality and Homogeneity test

Normality test was conducted on both control and experimental variables. Because the number of subjects was less than 50, Shapiro-Wilk test was used for normality test while Levene's test was used for homogeneity test. Table 3 shows normal distribution of all variables, as the p >0.05. The test in table 4 showed abnormal data in osteoblast, adipocyte, and empty lacuna (p <0.05), therefore Tamhane post-hoc analysis was used. Osteocyte variable showed normal variance (p >0.05), so the Bonferroni post-hoc analysis was used.

One-way ANOVA test

Comparative test for the numeric variables was performed using One-way ANOVA for data with normal distribution. Post-test average among groups was compared to find out the effect of each variable.

Table 5 shows significant mean differences between control and experimental groups (p = 0.000). Largest difference was found in adipocyte (p = 0.0000).

Table 6 shows that in each week of comparison, the experimental groups have higher amount osteoblast compared to control groups (p < 0.05).

Table 7 shows that the experimental groups have lower amount of adipocyte compared to control groups in each week of comparison (p < 0.05).

Table 8 shows higher amount of osteocyte in experimental groups compared to control groups in each week of comparison (p < 0.05).

Table 9 shows that the study group treated with 4 mg of astaxanthin, 0.106 mg/kgBW/day dose, had less mean empty lacunae count compared to control group for 3 weeks straight. The mean difference is found to be statistically significant with p<0.05.

**Table 1** Frequency distribution of each group

|           | Week 1 | Week 2 | Week 3 |
|-----------|--------|--------|--------|
| Control   | 4(16.7%) | 4(16.7%) | 12(50%) |
| Experimental | 4(16.7%) | 4(16.7%) | 12(50%) |
| Total     | 24(100%) |        |        |

**Table 2** Variable average of osteoblast, osteocyte, adipocyte, and osteonecrosis incidence between control and experimental group during research

| Variable   | Week 1 Average (s,b) | Week 2 Average (s,b) | Week 3 Average (s,b) |
|------------|----------------------|----------------------|----------------------|
| Osteoblast | Control 38 (1.43)    | 24.69 (0.83)         | 16.94 (1.28)         |
|            | Experimental 55.63 (2.17) | 68.94 (0.55) | 78.94 (0.55) |
| Osteocyte  | Control 16.69 (0.94) | 11.94 (1.65)        | 7.75 (1.40)          |
|            | Experimental 28.06 (1.26) | 35.88 (1.05) | 42 (0.20)           |
| Adipocyte  | Control 58.69 (1.18) | 88.75 (4.66)        | 111.63 (1.53)        |
|            | Experimental 27.50 (1.24) | 21.00 (1.48) | 14.63 (0.48)        |
| Empty lacuna | Control 18.5 (1.29) | 28.69 (0.24)      | 34.56 (0.31)         |
|            | Experimental 13.25 (0.91) | 7.94 (0.94) | 4.75 (0.79)          |

**Table 3** Normality test of variables using Shapiro-Wilk

| Variable   | Category   | Shapiro-Wilk |
|------------|------------|--------------|
|            | Statistic  | Df | Sig |
| Osteoblast | Control 1 week | 0.973 | 4 | 0.861 |
|            | Experimental 1 week | 0.782 | 4 | 0.074 |
|            | Control 2 weeks | 0.980 | 4 | 0.900 |
|            | Experimental 2 weeks | 0.801 | 4 | 0.103 |
|            | Control 3 weeks | 0.930 | 4 | 0.594 |
|            | Experimental 3 weeks | 0.801 | 4 | 0.103 |
| Osteocyte  | Control 1 week | 0.994 | 4 | 0.976 |
|            | Experimental 1 week | 0.997 | 4 | 0.989 |
|            | Control 2 weeks | 0.978 | 4 | 0.889 |
|            | Experimental 2 weeks | 0.980 | 4 | 0.889 |
|            | Control 3 weeks | 0.909 | 4 | 0.479 |
|            | Experimental 3 weeks | 0.945 | 4 | 0.683 |
| Adipocyte  | Control 1 week | 0.814 | 4 | 0.130 |
|            | Experimental 1 week | 0.952 | 4 | 0.726 |
|            | Control 2 weeks | 0.972 | 4 | 0.855 |
|            | Experimental 2 weeks | 0.998 | 4 | 0.995 |
|            | Control 3 weeks | 0.971 | 4 | 0.848 |
|            | Experimental 3 weeks | 0.863 | 4 | 0.272 |
| Empty lacuna | Control 1 week | 0.993 | 4 | 0.972 |
|            | Experimental 1 week | 0.950 | 4 | 0.714 |
|            | Control 2 weeks | 0.863 | 4 | 0.272 |
|            | Experimental 2 weeks | 0.994 | 4 | 0.976 |
|            | Control 3 weeks | 0.895 | 4 | 0.406 |
|            | Experimental 3 weeks | 0.940 | 4 | 0.653 |

**Table 4** Homogeneity test of variables using Levene’s test

| Variable   | Levene Statistic | df1 | df2 | sig |
|------------|------------------|-----|-----|-----|
| Osteoblast | 4.404            | 5   | 18  | 0.000 |
| Osteocyte  | 7.807            | 5   | 18  | 0.085 |
| Adipocyte  | 2.546            | 5   | 18  | 0.001 |
| Empty lacuna | 2.923          | 5   | 18  | 0.042 |
### Table 5  
*One-way ANOVA test of the control and experimental groups*  
|                          | Sum of squares | df | Mean square | F       | Sig    |
|--------------------------|----------------|----|-------------|---------|--------|
| **Osteoblast sum and average** |                |     |             |         |        |
| Between groups           | 12232.156      | 5  | 2446.431    | 1511.958| 0.000  |
| Within groups            | 29.125         | 18 | 1.618       |         |        |
| Total                    | 12261.281      | 23 |             |         |        |
| **Osteocyte sum and average** |              |     |             |         |        |
| Between groups           | 3776.336       | 5  | 755.267     | 544.814 | 0.000  |
| Within groups            | 24.953         | 18 | 1.386       |         |        |
| Total                    | 3801.289       | 23 |             |         |        |
| **Adipocyte sum and average** |            |     |             |         |        |
| Between groups           | 31565.076      | 5  | 6313.015    | 1285.138| 0.000  |
| Within groups            | 88.422         | 18 | 4.912       |         |        |
| Total                    | 31653.497      | 23 |             |         |        |
| **Empty lacuna**         |                |     |             |         |        |
| Between groups           | 2752.607       | 5  | 550.521     | 791.761 | 0.000  |
| Within groups            | 12.516         | 18 | 0.695       |         |        |
| Total                    | 2765.122       | 23 |             |         |        |

### Table 6  
*Tamhane post-hoc analysis of osteoblast variables of control and experimental groups*  
| Tamhane variable osteoblast | Group J                   | Mean difference (I-J) | p       | 95% interval | Confidence |
|----------------------------|----------------------------|-----------------------|----------|--------------|------------|
| Control 1 week             | Experimental 1 week        | -17.62500*            | 0.000    | -24.2551     | -10.9949   |
| Experimental 1 week        | Control 2 weeks            | 13.31250*             | 0.000    | 8.8847       | 17.7403    |
| Control 2 weeks            | Experimental 2 weeks       | -30.93750*            | 0.000    | -35.8306     | -26.0444   |
| Experimental 2 weeks       | Control 3 weeks            | 21.06250*             | 0.000    | 16.5483      | 25.5767    |
| Control 3 weeks            | Experimental 3 weeks       | -40.93750*            | 0.000    | -45.8306     | -36.0444   |
| Experimental 3 weeks       | Control 1 week             | 17.62500*             | 0.000    | 10.9949      | 36.0444    |
| Control 1 week             | Control 2 weeks            | 30.93750*             | 0.000    | 23.4470      | 38.4280    |
| Control 2 weeks            | Experimental 2 weeks       | -13.31250*            | 0.011    | -21.5497     | -5.0753    |
| Experimental 2 weeks       | Control 3 weeks            | 38.68750*             | 0.000    | 31.9700      | 45.4050    |
| Control 3 weeks            | Experimental 3 weeks       | -23.1250*             | 0.002    | -31.5497     | -15.0753   |
| Experimental 3 weeks       | Control 1 week             | -13.31250*            | 0.000    | -17.7403     | -8.8847    |
| Control 1 week             | Experimental 1 week        | -30.93750*            | 0.000    | -38.4280     | -23.4470   |
| Experimental 1 week        | Control 2 weeks            | -44.25000*            | 0.000    | -46.7651     | -41.7349   |
| Control 2 weeks            | Control 3 weeks            | 7.75000*              | 0.002    | 3.8390       | 11.6610    |
| Control 3 weeks            | Experimental 3 weeks       | -54.25000*            | 0.000    | -56.7651     | -51.7349   |
| Experimental 2 weeks       | Control 1 week             | 30.93750*             | 0.000    | 26.0444      | 35.8306    |
| Control 1 week             | Experimental 1 week        | 13.31250*             | 0.011    | 5.0753       | 21.5497    |
| Experimental 1 week        | Control 2 weeks            | 44.25000*             | 0.000    | 41.7349      | 46.7651    |
| Control 2 weeks            | Control 3 weeks            | 52.00000*             | 0.000    | 47.7446      | 56.2554    |
| Control 3 weeks            | Experimental 3 weeks       | -10.00000*            | 0.000    | -11.8325     | -8.1675    |
| Control 2 weeks            | Experimental 1 week        | -21.06250*            | 0.000    | -25.5767     | -16.5483   |
| Experimental 1 week        | Control 2 weeks            | -38.68750*            | 0.000    | -45.4050     | -31.9700   |
| Control 2 weeks            | Experimental 2 weeks       | -7.75000*             | 0.002    | -11.6610     | -3.8390    |
| Experimental 2 weeks       | Control 3 weeks            | -52.00000*            | 0.000    | -56.2554     | -47.7446   |
| Control 3 weeks            | Experimental 3 weeks       | -62.00000*            | 0.000    | -66.2554     | -57.7446   |
| Experimental 3 weeks       | Control 1 week             | 40.93750*             | 0.000    | 36.0444      | 45.8306    |
| Control 1 week             | Experimental 1 week        | 23.31250*             | 0.002    | 15.0753      | 31.5497    |
| Experimental 1 week        | Control 2 weeks            | 54.25000*             | 0.000    | 51.7349      | 56.7651    |
| Control 2 weeks            | Experimental 2 weeks       | 10.00000*             | 0.000    | 8.1675       | 11.8325    |
| Control 3 weeks            | Experimental 3 weeks       | 62.00000*             | 0.000    | 57.7446      | 66.2554    |
### Table 7  Tamhane post-hoc analysis of adipocyte variable in control and experimental groups

| Tamhane variable adiposity | Group I | Group J | Mean difference (I-J) | p       | 95% interval       | Confidence |
|----------------------------|---------|---------|-----------------------|---------|--------------------|------------|
|                            |         |         | Lower bound           |         | Upper bound        |            |
| Control 1 week             |         |         | 31.18750*             | 0.000   | 27.1791            | 35.1959    |
| Control 2 weeks            | -30.66250* | 0.009   | -47.7550              | -12.3700|
| Experimental 2 weeks       | 37.68750*  | 0.000   | 33.1276               | 42.2474 |
| Control 3 weeks            | -52.93750* | 0.000   | -57.6259              | -48.2491|
| Experimental 3 weeks       | 44.06250*  | 0.000   | 40.0746               | 48.0504 |
| Experimental 1 week        | -31.18750* | 0.000   | -35.1959              | -27.1791|
| Control 2 weeks            | -61.25000* | 0.001   | -78.7637              | -43.7636|
| Experimental 2 weeks       | 6.50000*   | 0.009   | 1.8957                | 11.1043 |
| Control 3 weeks            | -84.12500* | 0.000   | -88.8483              | -79.4017|
| Experimental 3 weeks       | 12.87500*  | 0.001   | 8.6157                | 17.1343 |
| Control 2 weeks            |         |         | 30.06250*             | 0.009   | 12.3700            | 47.7550    |
| Experimental 1 week        | 61.25000*  | 0.001   | 43.7363               | 78.7637 |
| Experimental 2 weeks       | 67.75000*  | 0.000   | 50.9307               | 84.5693 |
| Control 3 weeks            | -22.87500* | 0.017   | -39.5609              | -6.1891 |
| Experimental 3 weeks       | 74.12500*  | 0.001   | 54.7174               | 93.5326 |
| Experimental 2 weeks       | -37.68750* | 0.000   | -42.2474              | -33.1276|
| Experimental 1 week        | -6.50000*  | 0.009   | -11.1043              | -1.8957 |
| Control 2 weeks            | -67.75000* | 0.000   | -84.5693              | -50.9307|
| Control 3 weeks            | -90.62500* | 0.000   | -95.6206              | -85.6294|
| Experimental 3 weeks       | 6.37500*   | 0.028   | 1.0301                | 11.7199 |
| Control 3 weeks            | 52.93750*  | 0.000   | 48.2491               | 57.6259 |
| Experimental 1 week        | 84.12500*  | 0.000   | 79.4017               | 88.4843 |
| Control 2 weeks            | 22.87500*  | 0.017   | 6.1891                | 39.5609 |
| Experimental 2 weeks       | 90.62500*  | 0.000   | 85.6294               | 95.6206 |
| Experimental 3 weeks       | 90.00000*  | 0.000   | 91.4381               | 102.5619|
| Experimental 3 weeks       | -44.06250* | 0.000   | -48.0504              | -40.0746|
| Experimental 1 week        | -12.87500* | 0.001   | -17.1343              | -8.6157 |
| Control 2 weeks            | -74.12500* | 0.001   | -93.5326              | -54.7174|
| Experimental 2 weeks       | -6.37500*  | 0.028   | -11.7199              | -1.0301 |
| Control 3 weeks            | -97.00000* | 0.000   | -102.5619             | -91.4381|

### Table 8  Bonferroni post-hoc analysis of osteocyte variable in control and experimental groups

| Bonferroni variable osteocyte | Group I | Group J | Mean difference (I-J) | p       | 95% interval       | Confidence |
|-------------------------------|---------|---------|-----------------------|---------|--------------------|------------|
|                               |         |         | Lower bound           |         | Upper bound        |            |
| Control 1 weeks              |         |         | -11.37500*            | 0.000   | -14.1893           | -8.5607    |
| Control 2 weeks              |         |         | 4.75000*              | 0.000   | 1.9357             | 7.5643     |
| Experimental 2 weeks         | -19.18750* | 0.000  | -22.0018              | -16.3732|
| Control 3 weeks              | 8.93750*  | 0.000   | 6.1232                | 11.7518 |
| Experimental 3 weeks         | -25.31250* | 0.000  | -28.1268              | -22.4982|
| Experimental 1 week          |         |         | 11.37500*             | 0.000   | 8.5607             | 14.1893    |
| Control 2 weeks              |         |         | 16.12500*             | 0.000   | 13.3107            | 18.9393    |
| Experimental 2 weeks         | -7.81250*  | 0.000   | -10.6268              | -4.9982 |
| Control 3 weeks              | 20.31250* | 0.000   | 17.4982               | 23.1268 |
| Experimental 3 weeks         | -13.93750* | 0.000  | -16.7518              | -11.1232|

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### Table 8  Continues

| Bonferroni variable osteocyte | Mean difference (I-J) | p   | 95% interval Lower bound | Upper bound | Confidence |
|-----------------------------|-----------------------|-----|--------------------------|-------------|------------|
| Control 1 weeks             | Control 2 weeks       | -4.75000* | 0.000 | -7.5643 | -1.9357 |
| Control 1 weeks             | Experimental 1 weeks  | -16.12500* | 0.000 | -18.9393 | -13.3107 |
| Control 1 weeks             | Experimental 2 weeks  | -23.93750* | 0.000 | -26.7518 | -21.1232 |
| Control 1 weeks             | Control 3 weeks       | 4.18750*  | 0.001 | 1.3732  | 7.0018  |
| Control 1 weeks             | Experimental 3 weeks  | -30.06250* | 0.000 | -32.8768 | -27.2482 |
| Experimental 2 weeks        | Control 1 weeks       | 19.18750* | 0.000 | 16.3732 | 22.0018 |
| Experimental 2 weeks        | Experimental 1 weeks  | 7.81250*  | 0.000 | 4.9982  | 10.6268 |
| Experimental 2 weeks        | Control 2 weeks       | 23.93750* | 0.000 | 21.1232 | 26.7518 |
| Experimental 2 weeks        | Control 3 weeks       | 28.12500* | 0.000 | 25.3107 | 30.9393 |
| Experimental 2 weeks        | Experimental 3 weeks  | -6.12500* | 0.000 | 8.9393  | -3.3107 |
| Control 3 weeks             | Control 1 weeks       | 5.25000*  | 0.013 | 1.3237  | 9.1763  |
| Control 3 weeks             | Experimental 2 weeks  | -10.56250* | 0.006 | -15.3252 | -5.0498 |
| Control 3 weeks             | Control 3 weeks       | 10.56250* | 0.000 | 6.6328  | 14.4922 |
| Control 3 weeks             | Experimental 3 weeks  | 13.75000* | 0.000 | 9.7852  | 17.7148 |
| Experimental 3 weeks        | Control 1 weeks       | 25.31250* | 0.000 | 22.4982 | 28.1268 |
| Experimental 3 weeks        | Experimental 1 weeks  | 13.93750* | 0.000 | 11.1232 | 16.7518 |
| Experimental 3 weeks        | Control 2 weeks       | 30.06250* | 0.000 | 27.2482 | 32.8768 |
| Experimental 3 weeks        | Experimental 2 weeks  | 6.12500*  | 0.000 | 3.3107  | 8.9393  |
| Experimental 3 weeks        | Control 3 weeks       | 34.25000* | 0.000 | 31.4357 | 37.0643 |

### Table 9  Tamhane post-hoc analysis of empty lacunae variable for control group and treatment group

| Tamhane empty lacunae variable | Mean difference (I-J) | p   | 95% interval Lower bound | Upper bound | Confidence |
|--------------------------------|-----------------------|-----|--------------------------|-------------|------------|
| Control 1 week                 | Experimental 1 week   | 5.25000* | 0.013 | 1.3237  | 9.1763  |
| Control 1 week                 | Control 2 weeks       | -10.18750* | 0.006 | -15.3252 | -5.0498 |
| Control 1 week                 | Experimental 2 weeks  | 10.56250* | 0.000 | 6.6328  | 14.4922 |
| Control 1 week                 | Control 3 weeks       | -16.06250* | 0.001 | -20.9940 | -11.1310 |
| Control 1 week                 | Experimental 3 weeks  | 13.75000* | 0.000 | 9.7852  | 17.7148 |
| Experimental 1 week            | Control 2 weeks       | -5.25000* | 0.013 | -9.1763 | -1.3237 |
| Experimental 1 week            | Control 3 weeks       | -15.43750* | 0.000 | -18.8762 | -11.9988 |
| Experimental 1 week            | Experimental 2 weeks  | 5.31250*  | 0.003 | 2.2416  | 8.3834  |
| Experimental 1 week            | Control 3 weeks       | -21.31250* | 0.000 | -24.5400 | -18.0850 |
| Experimental 1 week            | Experimental 3 weeks  | 8.50000*  | 0.000 | 5.6460  | 11.3540 |
| Control 2 weeks                | Control 1 week        | 10.18750* | 0.006 | 5.0498  | 15.3252 |
| Control 2 weeks                | Experimental 1 week   | 15.43750* | 0.000 | 11.9988 | 18.8762 |
| Control 2 weeks                | Experimental 2 weeks  | 20.75000* | 0.000 | 17.1719 | 24.3281 |
| Control 2 weeks                | Control 3 weeks       | -5.87500* | 0.000 | -6.8353 | -4.9147 |
| Control 2 weeks                | Experimental 3 weeks  | 23.93750* | 0.000 | 21.0511 | 26.8239 |
| Experimental 2 weeks           | Control 1 week        | -10.56250* | 0.000 | -14.4922 | -6.6328 |
| Experimental 2 weeks           | Experimental 1 week   | -5.31250* | 0.003 | -8.3834 | -2.2416 |
| Experimental 2 weeks           | Control 2 weeks       | -20.75000* | 0.000 | 24.3281 | -17.1719 |
| Experimental 2 weeks           | Control 3 weeks       | -26.62500* | 0.000 | 29.9903 | -23.2597 |
| Experimental 2 weeks           | Experimental 3 weeks  | 3.18750*  | 0.033 | 0.2618  | 6.1132  |
DISCUSSION

The interpretation results of the statistically analyzed study data correspond to the hypothesis. The interpretation will be discussed to ascertain the factors that influence the result of this study.

Study Subjects

This study used mice as subject to determine the effect of astaxanthin on the number of osteocytes, osteoblasts, and adipocytes in femoral head osteonecrosis which is induced by alcohol. Mice were chosen due to its availability, low maintenance cost, and its small size. Aside from that, mice can breed quickly and which is rather advantageous in securing more subjects that match the inclusion criteria. Specifically, another advantage is the size of mice’s bone that made it easier to the study. The difference in sex affects the incidence of osteonecrosis. Male mice were chosen as study subject so as to avoid effects of estrogen on osteonecrosis. A study shows that estrogen has an influence on the incidence of osteonecrosis by increasing the risk of thrombosis due to coagulation process. In regard to the previously mentioned advantages, male mice were chosen as this study’s subject.

Astaxanthin’s Effect on Osteocytes

From this study, it was found that the number of osteocytes was higher in mice treated using 4 mg of astaxanthin with dosage 0.106 mg/kg BW/day for 3 weeks straight. The difference was also proven to be significant when compared to control group without astaxanthin treatment with p=0.000.

Post-hoc test performed in this study showed significant difference on osteocyte count after 3 weeks of astaxanthin treatment. The average count of osteocytes kept increasing during the second and third week of treatment, which was also proven to be statistically significant with the value of p<0.05. This result is attributed to the antioxidant effect of astaxanthin which is 10 times higher than other carotenoids, such as lutein, lycopene, alpha-tocopherol, alpha-carotene, and beta-carotene. Astaxanthin can prevent the creation of free radicals such as catalase, SOD, and hydrogen peroxide that hinder the release of inflammatory mediators, such as TNF-a, IL-1b, IL-6 and IL-10, which in turn will result in the inhibition of iNOS stimulation and nitric oxide (NO) production. Increase in iNOS expression, that is observed in osteonecrosis group, may be the reason for high NO production. This high production is marked by apoptosis of osteoblast, osteocyte, and marrow cell. Thus, alongside the decrease of NO level, the lowering of osteocyte and osteoblast level can be achieved.

Effect of NO in reducing VEGF induced by hypoxic condition through cGMP mechanism indicates NO as endogen inhibitor of hypoxic and non-hypoxic expression of VEGF in vivo. The statement is in line to another study regarding angiogenic growth factor which showed that local administration of VEGF will increase defect healing in bone. Neovascularization that occurs will help the transport of inflammatory cells, fibroblasts, and growth factors to the injury site. Yang et al. in 2014, shows that implantation of co-transfected bFGF and collagen gene on a necrotic femoral head in animal subject results in angiogenesis repair on bone tissue.

Astaxanthin can also increase the number of osteocytes through inhibiting AMPK, increasing PPAR g, and reducing leptin, which indirectly result in formation of osteocytes from preosteoblast. New research displays the Wnt signaling pathway which is involved in regulation of bone mass homeostasis. This pathway is also regulated by alcohol-induced ROS formation. Astaxanthin treatment will indirectly regulate Wnt signaling in osteocyte and osteoblast homeostasis.

### Table 9

**Tamhane empty lacunae variable**

| Group | Control 3 weeks | Experimental 3 weeks |
|-------|-----------------|----------------------|
| Group I | Control 1 week | Group J | Mean difference (I-J) | p | 95% interval | Confidence |
|       | 16.06250* | Control 1 week | 0.001 | 11.1310 | 20.9940 |
|       | 21.31250* | Experimental 1 week | 0.000 | 18.0850 | 24.5400 |
|       | 5.87500* | Control 2 weeks | 0.000 | 4.9147 | 6.8353 |
|       | 26.62500* | Experimental 2 weeks | 0.000 | 23.2597 | 29.9903 |
|       | 29.81250* | Experimental 3 weeks | 0.000 | 27.1241 | 32.5009 |
|       | -13.75000* | Control 1 week | 0.000 | -17.7148 | -9.7852 |
|       | -8.50000* | Experimental 1 week | 0.000 | -11.3540 | -5.6460 |
|       | -23.87500* | Control 2 weeks | 0.033 | -6.1132 | -0.2618 |
|       | -3.18750* | Experimental 2 weeks | 0.000 | -32.5009 | -27.1241 |
|       | -29.81250* | Control 3 weeks | 0.000 | -32.5009 | -27.1241 |
**Nuclearβ-catenin** act as transcription coactivator that interacts with the transcription factor of T-cell factor (Tcf)/Lymphoid enhancer factor (Lef) family to regulate gene expression. LRP5 removal in mice results in osteopenia, whereas over-expression of LRP5 will increase bone mass and reduce osteoblast apoptosis.

**Astaxanthin’s Effect on Osteoblasts**

In this study, it was found that the number of osteoblast on mice treated using 4 mg of astaxanthin with a dose of 0.106 mg/kgBW/day for 3 weeks straight. The difference in cell number compared to the control group without astaxanthin treatment was proven statistically significant with p=0.000.

Post-hoc test on this study resulted in significant difference in osteoblast count after treatment using astaxanthin for 3 weeks. The mean osteoblast count kept increasing on second and third week and has been proven to be statistically significant with p<0.05.

This finding is parallel to other study regarding effects of alcohol on bone, which shows that the decrease in osteoblast count and function correlates to increase in osteoclast number and cause increase in bone reabsorption. Aside from that, exposure to alcohol results in osteogenesis marker suppression in MSC during osteoblast formation. Antioxidant effect of astaxanthin also exerts influence on osteoblast production and biological pathway which is used by osteocytes. Aside from that, astaxanthin can also prevent the forming of free radicals, such as SOD and hydrogen peroxide, and inhibit the release of inflammatory mediators that will lower NO production. Higher NO production will inhibit proliferation of osteoblast and osteocytes. Suppression of AMPK, increase in PPAR γ, degradation of leptin, increase in VEGF through PI3K pathway alongside expression of HIF-1 alpha, and regulation of Wnt signaling happen through the same process as osteocyte.

**Astaxanthin’s Effect on Adipocyte Cells**

This study found that the number of adipocytes was lower in mice treated using 4 mg of astaxanthin with dosage 0.106 mg/kgBW/day for 3 weeks. The difference was also proven to be significant when compared to control group without astaxanthin treatment with p=0.000.

Post-hoc test performed in this study showed significant difference on adipocyte count after 3 weeks of astaxanthin treatment, which was proven to be statistically significant with value of p<0.05.

Antilipid drugs, for example Pitavastatin has an effect to inhibit adipogenesis. Similarly, oral vitamin E also reduces adipocyte count die to corticosteroid. Supplement effect of astaxanthin result in smaller visceral adipocyte size with decline in free fatty acid level and rise in HDL concentration. This result highlighted the function of astaxanthine to lower fat level and fat abnormality.

The result of this research fall in line with the role of astaxanthin in the induction of PPAR g target gene which will modulate PPAR g and finally increase the level of αP2 and lipoprotein lipase. Astaxanthin also increase osteogenic differentiation potential and adipogenic stem cell. These stem cells also show alarming calcium deposit. The result was consistent to the overexpression of genes that are connected to osteogenesis (osteoclast, RXR, and osteopontine) and genes that are involved in adipogenesis (AP and PPAD g) after treatment using Astaxantin. This process will decrease adipocyte count through inhibition of adipogenesis.

**Astaxanthin’s Effects on the Occurrence of Osteonecrosis**

The incidence of osteonecrosis in this study was determined by the presence of empty lacunae. The mean total of empty lacunae occurred in control group during the third week (34.56±0.31). On the other side, the mean total of empty lacunae in treatment group during the third week was the lowest amount (4.75±0.79).

In this study, it was found that the mean number of empty lacunae on mice treated using 4 mg of astaxanthin with dose of 0.106 mg/kgBW/day for 3 weeks straight. The difference compared to the control group without astaxanthin treatment was proven statistically significant with p=0.000.

Post-hoc test on this study resulted in significant difference in mean total of empty lacunae count after treatment using astaxanthin for 3 weeks. This has been proven to be statistically significant with p<0.05.

Increase in alcohol-induced oxidative stress, in which adipogenesis, osteogenesis, and angiogenesis disturbance occur and result in disturbance in blood circulation to the bone, is the etiology of alcohol exposure femoral head osteonecrosis. If adipogenesis is inhibited, angiogenesis and osteogenesis will in turn increase and reduce alcohol exposure osteonecrosis. Alcohol consumption causes increase in ROS production which will turn into inflammation response by releasing IL-1b and TNF-a. This process stimulates iNOS which later increase NO level that resulted in the rise of intracellular cGMP, osteoblast apoptosis, and osteocyte apoptosis. Hypoxemia injury stimulates HIF-1α that increase expression of VEGF. However, VEGF regulation is diminished by stimulation of intracellular cGMP and resulted in reduced angiogenesis that will lead to less stimulation to repair necrotic
area. Those mechanism explained the collapse of femoral head.

Statin has an anti-lipid effect on adipogenesis and can reduce the incidence of osteonecrosis. Oral vitamin E can also reduce the incidence of corticosteroid induced osteonecrosis. Astaxanthin which is an oxidized carotenoid works as modulator of PPAR g that effectively increase mRNA levels of aP2, lipoprotein lipase, and leptin in order to regulate and hinder adipogenesis. It will in turn reduces the number of adipocytes and prevent formation of fat embolism that may damage the blood vessel. Damage in blood vessel may cause hypoxemia injury that may lead to osteonecrosis.

Antioxidant from astaxanthin also influence osteoblast and osteocyte. Astaxanthin can also prevent formation of free radicals, such as catalase, SOD, and hydrogen peroxide, and also inhibit the release of inflammatory mediators that may suppress NO production. High NO production hinders proliferation of osteoblast and osteocyte. Aside from that, astaxanthin suppress AMPK, increases PPAR g, reduces leptin, increases VEGF through PI3K pathway with expression of HIF-1 alpha and regulation of Wnt signaling pathway that ends up increasing the amount of osteocyte and osteoblast.

In accordance to previous statements, astaxanthin has strong potential as antilipid, immunomodulator, and antioxidant that suppress oxidative stress as the pathogenesis for alcohol-induced osteonecrosis. With decrease of adipogenesis and increase in osteogenesis and angiogenesis, then the incidence of alcohol exposure osteonecrosis will be decreased.

CONCLUSION

According to the study result of astaxanthin treatment on mice, it can be concluded that administration of astaxanthin results in increased osteocyte and osteoblast count in alcohol-exposed mice with femoral head osteonecrosis compared to those without astaxanthin administration. Administration of astaxanthin results in reduced adipocyte count incidence of osteonecrosis in the femoral head of alcohol-exposed mice with femoral head osteonecrosis compared to those without astaxanthin administration.

RECOMMENDATION

Astaxanthin can be used as a natural ingredient in prevention of the femoral head osteonecrosis. However, researches using non-animal samples or larger amount of samples are still needed to achieve clinical effect, especially on human.

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DISCLOSURE

The author reports no conflicts of interest in this work.

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