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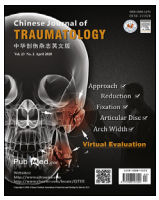

Original Article

Low expression of vascular endothelial growth factor and high serum level of cyclic guanine monophosphate as the risk factors of femoral head osteonecrosis in alcohol-exposed Wistar rat

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ABSTRACT

Purpose: Severe damage to the femoral head in patients with osteonecrosis has a high impact on morbidity. Despite early diagnosis, the treatment outcome is still unsatisfactory. This study aimed to explore the expression of vascular endothelial growth factor (VEGF) and cyclic guanine monophosphate (cGMP) serum level as the risk factors of femoral head osteonecrosis in alcohol-exposed Wistar rats.
Methods: This was an experimental study using randomized post-test only control group design, with samples using 10–14 weeks Wistar male rats. Rats were then divided into 6 groups: 3 groups without intervention, and 3 groups with intervention using 40% alcohol given perorally. Each one group from intervention and control group was euthanized by the end of the week for 3 consecutive weeks. Proximal femurs were examined under microscope for osteonecrosis, immunohistochemically for VEGF, and blood serum for cGMP levels.
Results: VEGF expression in the femoral head of alcohol-exposed Wistar rats was lower than those not exposed to alcohol ($p < 0.005$). Blood serum cGMP levels of alcohol-exposed Wistar rats were higher than those not exposed to alcohol ($p < 0.005$). The number of necrotic osteocytes in the femoral head of Wistar rats exposed to alcohol was greater than those not exposed to alcohol ($p < 0.005$). There are significant differences between VEGF, cGMP levels, and number of necrotic osteocytes in the control group and treatment at 1st, 2nd, and 3rd week ($p < 0.005$).
Conclusions: Based on the result of this study, VEGF and cGMP may be considered as diagnostic biomarkers for alcohol-induced femoral head osteonecrosis.

Introduction

Corticosteroids of alcohol-induced femoral head osteonecrosis comprises approximately one-third of the total non-traumatic osteonecrosis cases. The relationship between alcohol and osteonecrosis has been demonstrated previously in several studies. In vitro studies in rats and rabbits show that alcohol induces changes in bone marrow stromal cells to adipocytes. Apart from that, there is also an increase in serum triglycerides and cholesterol that will accumulate in the subchondral region of the femoral head. This buildup of intracellular fat cells will result in osteocyte cell death and inhibit the process of osteogenesis. The amount of alcohol required to increase the risk of osteonecrosis is not known yet, but one study revealed that the risk of osteonecrosis is increased in individuals with alcohol consumption of more than 400 mL per week.[1]

The occurrence of osteonecrosis has been studied both in humans and in experimental animals, and various factors including coagulation disorders, an oxidative stress were found as risks. Osteonecrosis of the femoral head can be triggered by changes in blood vessels. New research using animal models has successfully demonstrated the role of vascular endothelial growth factor (VEGF) 2 receptors in the process of angiogenesis and the formation of osteonecrosis.[2] VEGF can bind to its receptors and upregulate the angiogenesis process by giving signals to endothelial cells to undergo a process of proliferation, migration, and differentiation into new blood vessels. Anti-VEGF antibodies can also cause nonunion because angiogenesis also plays a major role in the process of

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fracture healing. VEGF has also been used to improve angiogenesis during surgery, as evidenced by increased blood flow in necrotic bones.[3] Nitric oxide (NO) plays a central role in complex apoptotic signaling pathways and plays a major role in bone function. NO is a free radical messenger molecule produced from the amino acid l-arginine by the enzymatic action of NO synthase (NOS).[4] NO is an important regulatory factor in the process of apoptosis and can be an inducer or inhibitor. A study by Chung et al.[5] states that NO plays two roles in cell apoptosis process. The role of this complex NO is generally related to NO concentrations. A high NO concentration will activate the caspase protein family and start several events and reactions that will result in the apoptosis process. On the other hand, low levels of NO concentration or physiology can act as anti-apoptotic agents through direct inhibition rather than caspase activity or by activating protective genes such as heat shock protein. NO plays a role as the main mediator of bone function, as well as a modulator of bone cell apoptosis.[6]

VEGF is a mitogen vascular endothelial cell required for the normal vascular formation and was first identified as an endothelial growth factor of bovine pituitary follicular cells by Ferrara.[7] VEGF also increases neovascularization and vascular permeability.[8,9] Of all isoforms, VEGF-A is the dominant factor in the regulation of angiogenesis and endothelial cell growth. Vasculogenesis and angiogenesis are triggered by an increase in local and systemic VEGF levels, thus enabling osteogenic substrate mobilization, pericyte stem cells, and differentiated mesenchymal stem cells (MSC). Angiogenesis is an important component of bone growth and repair. The process depends on the adequacy of the formation of new capillaries from existing blood vessels. One stimulus that causes high VEGF expression is hypoxia. This decrease in oxygen pressure will stimulate vascular endothelial cells to produce VEGF resulting in angiogenesis, which is an important process that also occurs in tendon repair and remodeling of the tendon graft.[10] Ghiso et al.[11] showed the relationship between NO and VEGF through a mechanism that depends on cyclic guanosine monophosphate (cGMP). The general effect of NO-mediated mainly through intracellular cGMP and an increase in cGMP levels correlated with a decrease in VEGF levels. This shows the potential role of inhibition of VEGF expression by NO in biological processes.

Severe damage to the femoral head in patients with osteonecrosis has a high impact of morbidity. Although identification and early intervention have been carried out, the damage that has occurred has not yet been repaired. Prevention of the osteonecrosis process is the ideal strategy for handling this disease. Therefore, a diagnostic test in osteonecrosis is important. VEGF and cGMP present as promising diagnostic tests for osteonecrosis. The earlier the detection, the faster treatment can be given, and the morbidity can be minimalized. Therefore, this study aimed to investigate the expression of VEGF, cGMP serum level, and amount of osteocyte necrosis in correlation with alcohol-induced femoral head osteonecrosis in Wistar rat.

Methods

This study was an experimental study designed using the experimental randomized post-test control only group design. This research has been approved by Research Ethics Committee of Faculty of Medicine of Udayana University with ethical clearance number of 1399/UN14.2.2/PD/KEP/2018. The research was conducted in four places, namely: (1) Pharmacology Laboratory of the Faculty of Medicine, Udayana University, Bali, as a place for treating and maintaining rats, as well as a place for harvesting specimens from rats. (2) Histology Laboratory of the Faculty of Medicine, Udayana University, as an immunohistochemical examination site (number of VEGF expressions on the head of the femur Wistar rat). (3) Clinical Pathology Laboratory, Faculty of Medicine, Udayana University, as a place and examination of venous blood samples (cGMP rat). (4) Veterinary Pathology Laboratory of the Faculty of Veterinary Medicine, Udayana University, as a place for histopathology examination (number of necrotizing osteocytes). The research was conducted from December 2018 to January 2019. The samples included for this study are male Wistar rats aged 10–14 weeks, with body weight of 200–250 g, in a good health condition, and active without limping. Sick Wistar rats and those with appetite problem, defects, or extremity deformities were excluded from the research. The sample size was calculated using Federer's formula.[12]

Based on the formula above, the number of samples needed in this study was 5 experimental animals for each group or a total of 30 rats. Simple randomization technique was used to select appropriate samples because the population is relatively homogeneous. The steps in conducting this research were as follows: (1) Thirty male rats from Wistar species aged between 10 and 14 weeks were selected. (2) Rats were then divided into 6 groups, where P0, P1 and P2 groups received no intervention, while group P3, P4 and P5 received intervention in the form of 40% intraoral alcohol. (3) The administration of oral alcohol is carried out on the morning at 9:00 a.m. for 3 weeks. (4) The rats were grounded in the Veterinary Laboratory of the Faculty of Veterinary Medicine, Udayana University with a cage size of 30 cm × 20 cm, a temperature of 20 °C–25 °C, and a normal given diet in the form of pellets containing protein (20%), fat (5%), carbohydrates (45%), crude fiber (5%), vitamins, and minerals. Each rat received a diet of 12 g–20 g per day. Drinking water was provided as needed. (5) The weight of each rat was measured every week during the research. (6) On the last day of the first week, rats from P0 and P3 groups were euthanized using 200 mg/kg intramuscular ketamine injection, and their femoral heads were harvested. The same treatment was conducted for group P1 and P4 by the end of the second week, and for group P2 and P5 by the end of the third week. Their femoral heads were then examined histopathologically to assess the amount of osteocyte necrosis. Eye puncture was performed to collect blood samples from ophthalmic veins for cGMP level examination. Finally, the rest of the rats' bodies were buried. The flow diagram of study as shown in Fig. 1.

Concentration and dose of alcohol

The alcohol used is 40% alcohol with a dose of 0.5 mL per day given intraorally obtained from the calculation of the specific gravity of alcohol. It is known that the specific gravity of alcohol is 0.78 g/mL. The dose of alcohol used to induce osteonecrosis is 12.94 g/kg/day, with a rat weight of 200 g so an alcohol dose of 2.6 g/day is obtained. With the calculation of the specific gravity formula, the volume of alcohol obtained is 0.3 mL, which is a calculation with 100% pure alcohol, so in this study a 40% alcohol dose was used with an alcohol dose amount of 0.5 mL per day.

Making of histopathological bone preparations

The rats were euthanized using Ketamine injection 3 times the anesthetic dose which is 3 times 44 mg/kg, then the second proximal femur was taken after the rat died. The two bones were then fixed for 1 week with 10% formalin-0.1 M phosphate buffer pH 7.4. Bone samples were then decalcified with 25% formic acid for 3 days then neutralized with 0.35 M sodium sulphate for 3 days. The specimens were implanted in paraffin, cut into 4 mm sections and painted with hematoxylin and eosin. The part of the femur bone of the rat examined is the trabecular head of the femur. For immunohistochemical examination of femoral head, the bone trabecular preparations were taken from the anterosuperior part of the lateral femoral head by calculating the number of VEGF expressions. For

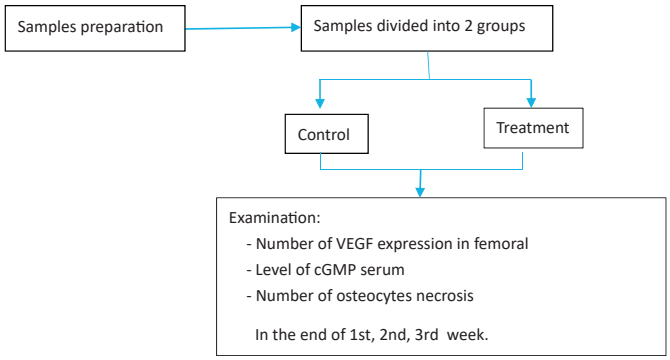

Fig. 1. Flow diagram of study.

histopathological examination of femoral head, the bone trabecular preparations are taken from the anterosuperior lateral cap of the femur by calculating the number of necrotic osteocytes. The rest of the body and tissues of mice that are not used are then buried or burned.

Immunohistochemical staining of VEGF

Deparaffinized sections were digested with 0.1% trypsin (Sigma, USA) and 0.1% $CaCl_2$ in phosphate-buffered saline (PBS) for 15 min at 37 °C and washed with deionized water. Endogenous peroxidase activity was deactivated by immersing sections in 3% hydrogen peroxide in 0.01 M PBS for 10 min at 37 °C, and rinsing several times in PBS. Sections were blocked with 10% bovine serum in PBS for 30 min at room temperature to reduce nonspecific binding, and incubated overnight at 4 °C with a primary antibody. Primary antibodies against VEGF and CD31 (Upstate Biotechnology, Lake Placid, NY, USA) were used. The VEGF primary antibody is a mouse anti-rabbit antibody that recognizes 121, 165, and 189 amino acid isoforms of rabbit VEGF. A goat anti-mouse IgG biotinylated antibody diluted in a buffer (1:250) was used as a secondary antibody. Sections were further incubated with the biotinylated anti-mouse IgG and streptavidin peroxidases for 30 min, respectively, at 37 °C. Then sections were thoroughly rinsed in PBS with shaking following each step. Immunoreactivity was determined by incubating the sections in a chromogen solution containing diaminobenzidine (DAB) and 0.1% hydrogen peroxide in the dark, followed by counterstaining with hematoxylin. The sections were dehydrated in alcohol and mounted using Permount. Negative control sections were processed using similar steps as described above but were incubated.[13]

Wistar rats blood serum taking

Examination of blood samples was carried out by taking eye vein blood by 2 mL by inserting a hematocrit microstructure into one eye of the rat. The blood is channeled through the micro hematocrit and then directly accommodated in the test tube. Taking blood samples is done shortly after the rat is sedated. This sample was then sent to the Clinical Pathology Laboratory of Sanglah Hospital to undergo cGMP examination. The method used is enzyme linked immune sorbent assay (ELISA) technology. The kit used is rat cGMP (Cyclic GMP) ELISA. Material samples are analyzed within 2 h or stored in a storage temperature of −20 °C for a longer period of time. Furthermore, the collected data was analyzed statistically by SPSS for Windows version 22.0. The data obtained were analyzed by the following steps: (1) descriptive analysis to identify variables, (2) analysis of normality and homogeneity with Saphiro Wilk test and Levene's test, respectively, (3) inferential analysis with independent *t*-test for normal distribution. This study used a 95% confidence interval (*CI*) and $p < 0.05$.

Results

VEGF expression level

The mean VEGF level in the 1st week control group was 39.30 ± 1.83 pg/mL, while the VEGF level in the 2nd week control group was 40.41 ± 1.53 pg/mL, and in the 3rd week control group was 41.10 ± 3.09 pg/mL. The mean VEGF level in 1st week treatment group was 16.87 ± 0.94 pg/mL, while the average VEGF level in 2nd week treatment group was 11.35 ± 1.60 pg/mL, and in the 3rd week treatment group was 5.26 ± 1.15 pg/mL. VEGF levels in the control group were highest at 3rd week and in the treatment group, the highest in the first week. Higher VEGF levels were in the control group than in the treatment group in 3 weeks of sampling. One way Anova test value of $p < 0.005$, then Ho is rejected, so there are significant differences between the VEGF levels of the control groups and treatment at 1st, 2nd and 3rd week (Table 1).

In the post-hoc test with least significant difference (LSD) test it was found that at 1st week, the treatment group had a significant difference in VEGF levels compared to the control group, with a $p < 0.005$. At 2nd week, the treatment group had a significant difference in VEGF levels compared to the control group, with a $p < 0.005$. At 3rd week, the treatment group had a significant difference in VEGF levels compared to the control group, with a $p < 0.005$. At 1st week, between the control group and the treatment group, the $p < 0.005$, 95% *CI* (20.0473–24.8087). At 2nd week, between the control group and the treatment group, the $p < 0.005$, 95% *CI* (26.6813–25.9227). At 3rd week, between the control and the treatment group, the $p < 0.005$, 95% *CI* (33.4593–38.2207). The immunohistochemistry of VEGF is shown in Fig. 2.

cGMP level

The average percentage of cGMP levels in the 1st week control group was 11.2520 ± 1.52191 pmoL/mL, in the 2nd week control group, it was 11.0960 ± 0.92037 pmoL/mL, and in the 3rd week control group, it was 11.3020 ± 1.70123 pmoL/mL. Average percentage level cGMP in the 1st week treatment group was 11.9740 ± 0.99213 pmoL/mL; the second-week treatment group was 15.9120 ± 1.90223 pmoL/mL, in the 3rd week treatment group was 20.0280 ± 1.62468 pmoL/mL. The highest level of cGMP was an examination at 3rd week, both in the treatment and control groups. cGMP levels were higher in the treatment group than in the control group in 3 weeks of sampling. One way Anova test $p < 0.005$, then ho is rejected, so there are significant differences between the cGMP levels of the control groups and treatment at 1st, 2nd and 3rd week (Table 2).

In the post-hoc test with LSD test it was found that at 1st week, the treatment group had a significant difference in VEGF levels compared to the control group, with a $p < 0.005$. At 2nd week, the treatment group had a significant difference in VEGF levels compared to the control group, with a $p < 0.005$. At 3rd week, the treatment group had a significant difference in VEGF levels

Table 1
Average VEGF expression levels in each group (mean ± SD).

Follow-up	VEGF expression level (pg/mL)		*p* value
	Control ($n = 15$)	Treatment ($n = 15$)	
1 week	39.30 ± 1.83	16.87 ± 0.94	< 0.005
2 weeks	40.41 ± 1.53	11.35 ± 1.60	
3 weeks	41.10 ± 3.09	5.26 ± 1.15	

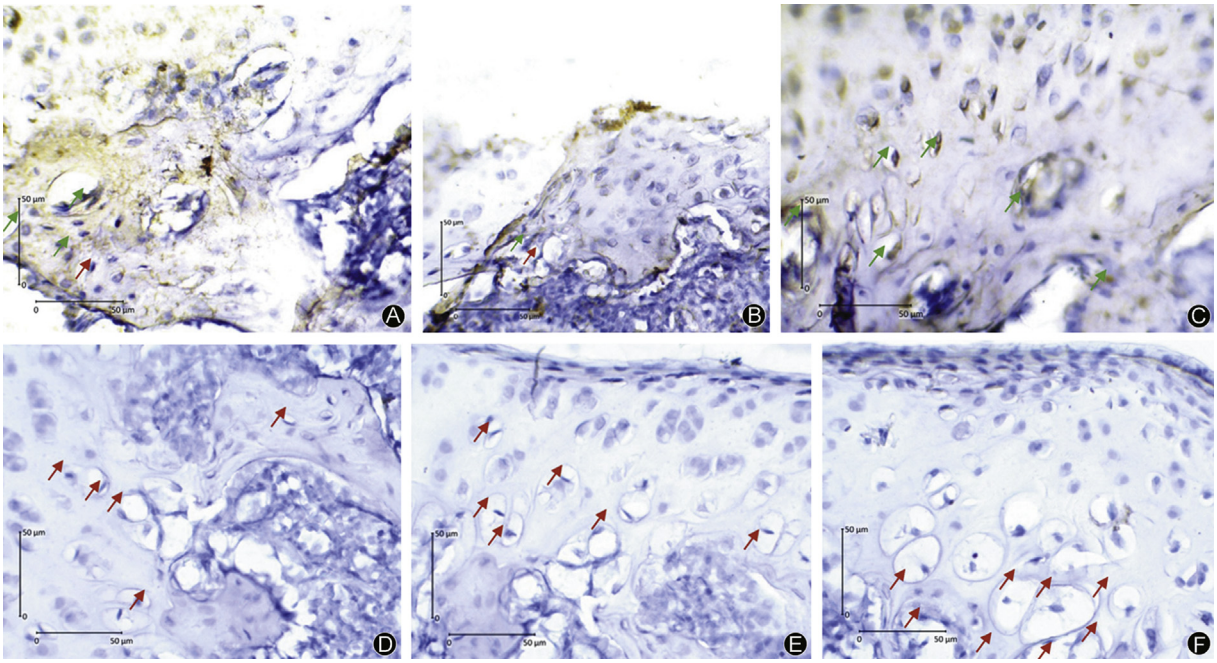

Fig. 2. Vascular endothelial growth factor: (A) 1st week control group, (B) 2nd weeks control group, (C) 3rd weeks control group, (D) 1st week alcohol group, (E) 2nd weeks alcohol group, (F) 3rd weeks alcohol group. Green arrows shows positive of VEGF staining (A, B and C) and red arrows shows negative or minimal VEGF staining (D, E and F).

compared to the control group, with a $p < 0.005$. At 1st week, between the control group and the treatment group, the $p = 0.000$, 95% *CI* (20.0473–24.8087). At 2nd week, between the control group and the treatment group, the $p < 0.005$, 95% *CI* (26.6813–25.9227). At 3rd week, between the control and the treatment group, the $p < 0.005$, 95% *CI* (33.4593–38.2207).

Number of osteocytes necrosis

The mean percentage of osteocytes necrosis in the 1st week control group was 12.4000 ± 1.14018 cells/field, while the mean number of osteocytes necrosis in the 2nd week control group was 12.8000 ± 0.83666 cells/field and in the 3rd week control group was 12.2000 ± 1.09545 cells/field. The average proportion of osteocytes necrosis in the 1st week treatment group was 15.6000 ± 1.67332 cells/field, while the mean number of osteocytes necrosis in the 2nd week treatment group was 33.2000 ± 2.58844 cells/field and in the 3rd week treatment group was 41.6000 ± 2.07364 cells/field. The number of osteocytes necrosis in the control group was highest at 2nd week and in the treatment group the highest at 3rd week. Higher osteocytes of necrosis were present in the treatment group than in the control group in 3 weeks of sampling. One way Anova test $p < 0.005$, then ho is rejected, so there is a significant difference between the number of osteocytes necrosis in the control and treatment groups at 1st, 2nd and 3rd week. (See Table 3).

In the post-hoc test with the LSD test it was found that at 1st week, the treatment group had a significant increase in the number of osteocytes necrosis compared to the control group, with a p value of 0.006. At 2nd week, the treatment group had a significant increase in the number of osteocytes necrosis compared to the control group, with $p < 0.005$. At 3rd week, the treatment group had a significant increase in the number of osteocytes necrosis compared to the control group, with $p < 0.005$. At 1st week, between the control group and the treatment group, the $p = 0.006$, 95% *CI* (−5.3972 - (- 1.0028)). At 2nd week, between the control group and the treatment group, the $p < 0.005$, 95% *CI* (−22.5972 - (- 18.2028)). At 3rd week, between the control group and the treatment group, the $p < 0.005$, 95% *CI* (−10.6694 - (- 6.7826)).

Fig. 3 shows the histopathological appearance of the femoral head after hematoxylin and eosin staining in typical experiments. Empty lacunae within the necrotic bone trabeculae, bone marrow cells including adipocytic necrosis and an accumulation of cell debris in the medullary space in most areas of the femoral head were observed at 1–3 weeks in the alcohol group. Feeding with the alcohol liquid diet for 3 weeks resulted in the formation of appositional bone around the necrotic bone trabeculae and, as part of the repair process, the deposition of fibrous and granulation tissue in the medullary space (Fig. 3). Additionally, partial appositional

Table 2
Average cGMP levels in each group (mean ± SD).

Follow-up	cGMP Level (pmol/mL)		*p* value
	Control ($n = 15$)	Treatment ($n = 15$)	
1 week	11.2520 ± 1.52191	11.9740 ± 0.99213	< 0.005
2 weeks	11.0960 ± 0.92037	15.9120 ± 1.90223	
3 weeks	11.3020 ± 1.70123	20.0280 ± 1.62468	

Table 3
Mean number of osteocytes necrosis in each group (mean ± SD).

Follow-up	Amount of osteocytes necrosis (cells/field)		*p* value
	Control ($n = 15$)	Treatment ($n = 15$)	
1 week	12.4000 ± 1.14018	15.6000 ± 1.67332	< 0.005
2 weeks	12.8000 ± 0.83666	33.2000 ± 2.58844	
3 weeks	12.2000 ± 1.09545	41.6000 ± 2.07364	

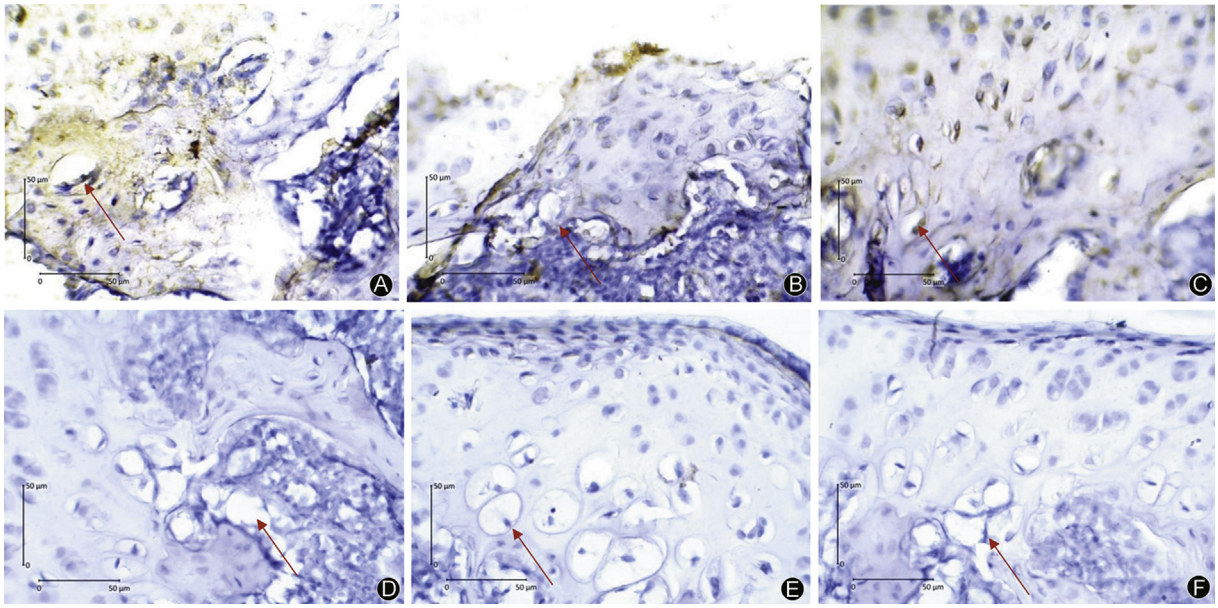

Fig. 3. Osteocytes necrosis: (A) 1st week control group, (B) 2nd weeks control group, (C) 3rd weeks control group, (D) 1st week alcohol group, (E) 2nd weeks alcohol group, (F) 3rd weeks alcohol group. The alcohol group rats show the formation of appositional bone around necrotic bone trabeculae, which represents part of the repair process, at 1, 2 and 3 weeks (red arrow).

bone thickening of the bone trabeculae and normal hematopoietic and fat cells were observed in the alcohol group.

Discussion

Effects of alcohol on VEGF

From this study, we found differences in VEGF levels between the control group and the treatment group, where VEGF levels in the treatment group were lower compared to the control group. Giving 40% alcohol as much as 0.5 mL every day for 3 weeks has shown a significant difference in VEGF levels with $p = 0.000$. The post-hoc test showed significant differences in VEGF levels between the control group and the treatment group, and there were also significant differences in the VEGF levels of the treatment group between 1st and 2 nd week, and between 2nd and 3rd week with $p < 0.005$.

This is contrary to one of the previous studies. Gu et al.[14] found that giving ethanol to chicken embryos with fibrosarcoma caused an increase of 1.2 to 2 times VEGF mRNA expression after exposure to ethanol compared to the control group, while Kasztelan-Szczerbinska et al.[15] found higher VEGF levels in people with alcoholic liver disease, and Heberlein et al.[16] show higher VEGF levels in patients with alcohol withdrawal syndrome. Research by Köttstorfer et al.[17] showed no association between chronic alcoholism and VEGF expression. This contradiction can be caused by the absence of previous studies examining specific VEGF expression in the femoral head tissue in patients with alcoholism. This can be a distinguishing factor between this research and previous studies. So, in future, other similar studies can be done to look for a mechanism for the reduction of specific VEGF expression in femoral head tissue with alcoholic patients.

Effect of alcohol on cGMP serum levels

Post-hoc tests in this study showed a significant difference in cGMP levels after administration of alcohol for 3 consecutive weeks. The cGMP levels continued to increase at weeks 2 and 3 and this proved to be statistically significantly different with $p < 0.05$. Alcohol is known to have a cytotoxic effect on bone cells by inducing cell death. Femoral head with a limited blood supply is more susceptible to alcohol accumulation and its metabolites, making it more prone to bone cell death in this area. Alcohol-induced cell death is thought to be associated with activation of NO. It is known that NO, especially when followed by high levels of prostaglandins, has toxic effects on osteoblasts and osteocytes, activates inflammatory mediators, and causes bone damage. NO induces the synthesis of cGMP, which is a cyclic nucleotide that is localized in bone cells. Because these two components have similarities in patterns of biological activity, an increase in NO will be followed by an increase in cGMP levels.[3,4] Thus, research by Calder et al.[6] and Teixeira et al.[18] support the results of our study, where high cGMP was associated with osteonecrosis in alcohol-treated mice.

However, this is not in line with some of the results of other studies. Koo et al.[19] stated that there was no relationship between femoral head osteonecrosis related to ethanol consumption with NO and cGMP levels. Other studies have shown that in inducing bone resorption, NO follows a different pathway with the collaboration of high levels of prostaglandins. There is also the opinion that NO is supposed to increase perfusion through the effects of internal vasodilators which lead to femoral head preservation.[18–21] However, Koo et al.[19] also conveyed the need for further research to ensure this, given the lack of research that was well designed in his study.

Effect of alcohol on the number of osteocytes necrosis

The post-hoc test in this study showed a significant difference in the value of osteocytes necrosis in the group that was given alcohol when compared with the control group that was not given alcohol in the first, second and third weeks with a value of $p < 0.005$. The value of osteocytes necrosis was found to increase since the administration of alcohol at week 1 to the peak at week 3 and this proved to be statistically significantly different from the value of $p < 0.005$. Alcohol is known to induce changes in osteoblast and osteoclast function, although there is still little research that discusses in detail the effects of alcohol on osteocytes.[22] A study by

Maurel et al.[23] stated that alcohol causes osteopenia, decreased trabecular density and cortical thickness, and massive osteocyte necrosis, associated with lipid accumulation into bone tissue.

High cholesterol levels and abnormal fat transport can also be associated with increased osteocyte necrosis, which will explain the decrease in bone mass.[23] The proportion of marrow lipids has been shown to increase in animals given alcohol. This increase can induce intra-osseous venous stasis and intra-osseous hypertension, thereby reducing fluid perfusion and blocking the venous system in the internal bone compartment. This can cause bone marrow to experience ischemia and lead to osteocyte necrosis.[23] Thus it can be concluded that alcohol administration is associated with an increase in the number of osteocytes necrosis, as one of the examinations to prove the occurrence of femoral osteonecrosis. In clinical terms, it is expected that the effects of alcohol consumption on the number of osteocyte necrosis and its danger to health can be proven, this can be an educational medium for practitioners to patients, to prevent the future incidence of femoral osteonecrosis.

Though this was a well-designed animal study, further researches in human are needed in order to provide better generalizability and therefore can be applied in human clinical practice, also to ensure the efficacy of VEGF level, cGMP level, and the amount of osteocytes necrosis as good markers to detect early risk of femoral head osteonecrosis in alcoholic patients.

By obtaining significant differences in the levels of VEGF, cGMP, and the number of osteocytes necrosis between rats exposed to alcohol and not exposed to alcohol, it is to be expected that clinically and practically the clinicians can be considered to do those examination for early detection of risk of femoral head osteonecrosis in alcoholic patients.

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Nil.

Ethical Statement

This research has been approved by Research Ethics Committee of Faculty of Medicine of Udayana University with ethical clearance number of 1399/UN14.2.2/PD/KEP/2018.

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Declaration of Competing Interest

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contribution

All authors contributed equally to the work.

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