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Vitamin C Administration Method On Osteoblast Healing In Rats Femur Bone

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ABSTRACT
Bone, as an important organ in the body, has a fundamental role in supporting biomechanical functions, carrying out the hematopoiesis process, and maintaining calcium homeostasis. The cellular components of bone involve various types of cells, including osteogenic precursor cells, osteoblasts, osteoclasts, osteocytes, and hematopoietic elements from the bone marrow. Vitamin C, or ascorbic acid, is a water-soluble vitamin that plays an important role in the formation of collagen, skin, tendons, ligaments, blood vessels, as well as in wound healing and maintenance of cartilage, bones, and teeth. This study aims to analysed mean value and difference in the number of osteoblast cells in the femur of rats exposed to alcohol between groups receiving vitamin C. Based on the results of the One Way ANOVA test, the p value = 0.018 <0.05 was obtained, which indicated a significant difference in the average osteoblasts between groups. Furthermore, in the Post Hoc test, a difference in the mean osteoblasts was found between groups T3 and T5 with a p value = 0.029 < 0.05 and between groups T4 and T5 with a p value = 0.044 < 0.05. Conclusion: Administration of vitamin C can affect the increase in the number of osteoblast cells in the bone healing process in mice exposed to alcohol.

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INTRODUCTION

Alcohol not only contributes to increased risk of bone damage, but can also worsen the fracture healing process. Excessive alcohol consumption can inhibit fracture healing, increase the likelihood of complications, and increase medical costs. Therefore, it is important to find an effective approach to speed up the recovery process in individuals who consume large amounts of alcohol. In addition, other factors such as age, existing medical conditions, and use of drugs also play a role in slowing fracture healing (Hendrawati et al., 2023).

In addition to age and comorbid conditions, nutrition also plays an important role in the fracture healing process. Patients with fractures need to maintain optimal nutritional status by ensuring adequate intake of calcium, phosphorus, protein, and vitamins C and D, which contribute greatly to supporting bone recovery. Other factors that affect fracture healing include local and systemic elements. Local factors such as infection, the presence of

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tumors or malignancies, and soft tissue injuries can inhibit healing. On the other hand, systemic factors such as smoking habits, diabetes mellitus, nutritional deficiencies, advanced age, use of certain drugs, and hormonal disorders can also slow down the healing process (Handono et al., 2022).

Systemic factors, such as long-term use of corticosteroids, can cause osteoporosis and increase the risk of fractures. This condition is caused by inhibition of IGF-1 and TGF-β production. In addition, smoking and excessive alcohol consumption also have a significant negative impact on the fracture healing process. Individuals who smoke or consume alcohol excessively tend to be more susceptible to nonunion and delayed union than those who do not have these habits (Wilaksono, 2022).

In animal model studies, experiments on rats given oral alcohol with 35% ethanol content for 6 weeks showed similar results to previous studies. Histopathological analysis of tibia bones revealed a decrease in bone surface containing active osteoblasts and a significant reduction in bone wall thickness.

Vitamin C, which acts as an antioxidant essential for collagen synthesis and bone healing, has shown potential in improving fracture healing impaired by alcohol consumption. Experiments with vitamin C administration in rats resulted in radiological and histological improvements in callus, indicating a positive effect on the healing process. Fracture healing in rat femurs exposed to alcohol faces significant challenges. Although it is known that alcohol inhibits fracture healing by suppressing osteoblast activity and proliferation, the extent to which vitamin C supplementation can increase bone callus diameter and osteoblast number under these conditions is still not fully understood. Studies that specifically evaluate whether vitamin C supplementation can improve fracture healing parameters in rats exposed to alcohol could potentially provide a more effective solution to this problem.

Literature Review

Bone Anatomy and Histology

Bone is an important organ that plays a crucial role in supporting biomechanical functions, facilitating the hematopoiesis process, and maintaining calcium balance in the body. As a viscoelastic biomaterial, bone consists of about 10% cells spread in a matrix that makes up the remaining 90%. Osteogenic cells are located in the periosteum and endosteum. The periosteum consists of an outer layer of dense connective tissue and an inner layer called the osteogenic layer. In contrast, the endosteum specifically contains osteogenic cells without connective tissue (Inayati & Isasih, 2023).

Bone is a specialized connective tissue that is metabolically active and continuously undergoes a process of re-modeling, in which old bone tissue is replaced by new bone tissue. This process allows bones to adapt to mechanical loads and stresses (Lauing et al., 2012). In addition to providing structural support and protection for body organs, bones also function as the main reservoir for calcium, magnesium, and phosphate, ions that are very important in various physiological functions of the body. Structurally, bones are composites consisting of cells embedded in an extracellular matrix stabilized by minerals, especially calcium hydroxyapatite, which provides strength and stiffness to bone tissue

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(Gokhale et al., 2001).

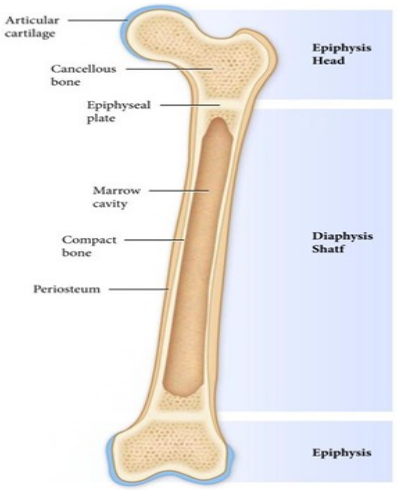

Figure 1. Anatomy of long bones

Osteoblasts and osteoclasts are present on the surface of compact and cancellous bone to form and resorb bone. Multipotent progenitor cells in the periosteum can differentiate into bone and cartilage cells, which is important when bone is injured (Lauing*et al.,*2012). Bones are of two main types, each with its own distinct function. Cortical (compact) bone forms the dense outer layer and serves primarily as a protective covering.

Bone types are divided into immature and mature bones. Immature bone, or woven bone, has collagen fibrils formed by osteoblasts, reducing strength but increasing flexibility. Immature bone can be found in the fetus, healing fractures, and certain pathological conditions. Meanwhile, mature bone consists of cortical (compact) bone and cancellous (trabecular, spongy) bone. Cortical bone, which makes up the majority of bones, has a Haversian system or osteons with osteocytes trapped in lacunae, communicating through canaliculi, and surrounded by Haversian canals (Apley, 2010).

This osteoid then undergoes mineralization to provide strength and stiffness to the bone. Some osteoblasts differentiate into osteocytes, while others remain on the surface of the periosteum and endosteum to continue their activities. In addition to forming bones, osteoblasts also regulate the formation of osteoclasts by releasing RANKL (receptor activator of nuclear factor kappa-B ligand), which supports the process of bone resorption by osteoclasts. Enzymes such as alkaline phosphatase and osteocalcin produced by osteoblasts play an important role in bone matrix mineralization, while also serving as markers of osteoblast activation and maturation.

Healing Process In Fractures

The fracture healing process generally consists of five phases, namely the hematoma phase, inflammation and cellular proliferation, callus formation, consolidation and remodeling (Salomon et al., 2010)

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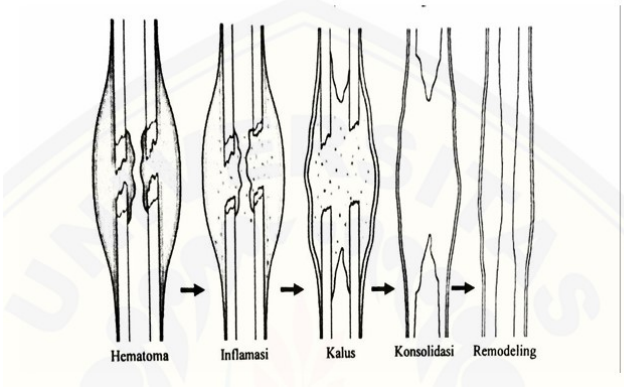

Figure 2. Bone healing process (Solomon et al., 2010)

The fracture healing process consists of several sequential phases, each with its own characteristics and important role in bone repair.

1. Hematoma Phase (1-3 days): In this early phase, blood clots and hematomas form around the fracture area. About 1-2 mm of the bone fragment ends up dying due to loss of blood supply.
2. Inflammatory and Cellular Proliferation Phase (starts 8 hours after fracture): An acute inflammatory reaction occurs with migration of inflammatory cells and proliferation of mesenchymal stem cells (MSCs) beneath the periosteum and in the medulla. The hematoma is slowly absorbed, new capillaries begin to grow, and the ends of the bone fragments are surrounded by cellular tissue that bridges the fracture.
3. Callus Formation Phase (4 weeks): Cells that proliferate in this phase are osteogenic and chondrogenic. Osteoblasts play a role in the resorption of dead bone, while woven bone or immature fibrous bone begins to form on the periosteal and endosteal surfaces. This phase marks clinical healing or union.
4. Consolidation Phase: In this phase, woven bone changes into lamellar bone through osteoclastic and osteoblastic activity. The accumulation of calcium salts in the callus makes the bone stiffer, although not yet strong enough to support normal loads.
5. Remodeling Phase: This phase lasts for several months to years, aiming to return the bone to its original anatomical shape. This process involves adjusting the bone structure to suit its biomechanical needs (Solomon et al., 2010).

Vitamin C

Vitamin C, or ascorbic acid, is a water-soluble vitamin that plays an important role in various physiological processes, including the formation of collagen, skin, tendons, ligaments, blood vessels, as well as in wound healing and the maintenance of cartilage, bones, and teeth. As an antioxidant, vitamin C also functions to suppress free radicals that can damage organs, tissues, and cells. Research shows that vitamin C can stimulate mesenchymal stem cells (MSCs) to differentiate into osteoblasts through the synthesis of type I collagen, which underlies the bone formation process (Srianuris, 2021).

The concentration of vitamin C in plasma and tissues is controlled by three main mechanisms: absorption, transport to tissues, and reabsorption and excretion by the kidneys. Vitamin C is absorbed in the gastrointestinal tract, and after entering the blood, it can be oxidized to dehydroascorbate. Vitamin C functions to donate electrons to the

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substrate, where it is oxidized to the ascorbyl radical. Two molecules of ascorbyl radicals can then break down into one molecule of ascorbate and one molecule of dehydroascorbic acid. Vitamin C transport occurs via the sodium vitamin C co-transporter (SVCT), which allows vitamin C in the form of dehydroascorbate to enter the mitochondria. In the mitochondria, dehydroascorbate is reduced back to ascorbate through the action of dehydroascorbate reductase and reduced glutathione. Ascorbate then exits the mitochondria, and further ascorbate synthesis takes place in the endoplasmic reticulum (Levani et al., 2021).

Vitamin C has the potential to enhance the fracture healing process by accelerating callus formation. The observed radiological and histological improvements indicate increased bone density and accelerated connective tissue formation. The important role of vitamin C in fracture healing is related to its ability to stimulate MSCs to differentiate into osteoblasts, which play a major role in new bone formation. In addition, vitamin C-mediated collagen type I synthesis is essential in the process of bone matrix mineralization, resulting in harder and stronger bones.

METHOD

Delayed fracture healing is a serious problem because it can cause high morbidity and high treatment costs. The delayed healing process can be influenced by various factors, both local and systemic. Consuming alcohol in large doses and over a long period of time can slow down the fracture healing process. Alcohol inhibits the differentiation of cells.*marrow stem cells*into osteoblasts, which reduces the number of osteoblasts that play a role in bone formation in the fracture area.

Vitamin C, or ascorbic acid, is a water-soluble vitamin that is essential for the formation of collagen, skin, tendons, ligaments, blood vessels, as well as in wound healing and the maintenance of cartilage, bones, and teeth. Vitamin C has great potential in improving the fracture healing process by accelerating callus formation. The improvements observed radiologically and histologically can indicate increased bone density and accelerated connective tissue formation, which supports a more efficient healing process.

Table 1. Operationalization of Variables (For Secondary Data)

Variables	Description	Data Used	Variable Units
Osteoblast Healing in Rat Femur Bone (Y)	Dependent Variable	Osteoblast Healing Data in Rat Femur Bones	Day
Vitamin C (X1)	Giving Vitamin C to Mice	Data on Vitamin C Consumption in Mice	mg

Table 2. Operationalization of Variables (For Primary Data)

Variables	Operational Definition	Indicator	Measurement Scale
Osteoblast (Y)	Number of Osteoblasts in Rat Femur Bones	Osteoblast Number Indicator in Rat Femur Bone	Unit
Vitamin C Dosage (X1)	Dosage of Vitamin C Given to Mice	Indicator of Vitamin C Dosage Given to Mice	mg

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The hypothesis in this study can be formulated as follows: There is a difference in the number of osteoblast cells in the femur of rats exposed to alcohol between the group receiving vitamin C and the group not receiving vitamin C. This hypothesis assumes that the administration of vitamin C can affect the number of osteoblast cells, which play a role in the fracture healing process, especially in fracture conditions that are hampered by alcohol exposure. This research is an experimental research designed using a Randomized post-test only control group design. The design of this research can be described by the following scheme:

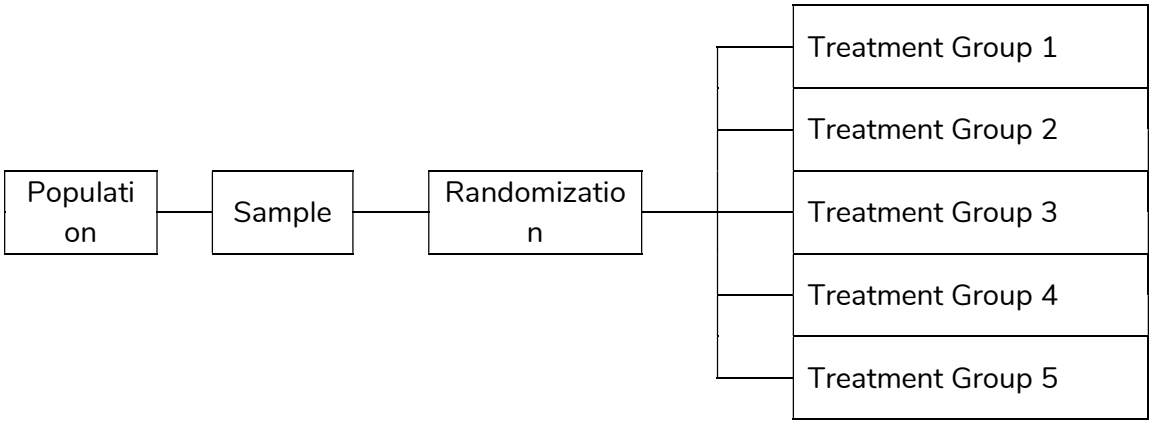

Figure 3. Research Design Scheme

Information
Treatment group 1: Group of mice given alcohol and vitamin C (normal)
Treatment group 2: Group of mice given Alcohol 1 ml 20%
Treatment group 3: Group of mice given vitamin C 250cc and Alcohol 1 ml 20%
Treatment group 4: Group of mice given Vitamin C 500cc and Alcohol 1 ml 20%
Treatment group 5: group of mice given 750cc of vitamin C and 1 ml of 20% alcohol

1. Descriptive analysis aimed to determine the mean value of femoral bone osteoblasts in rats exposed to alcohol between groups receiving vitamin C.
2. Bivariate Analysis. The bivariate analysis used was the One Way Anova Test to determine the difference in the mean of femoral bone osteoblasts in rats exposed to alcohol between groups receiving vitamin C. Before the One Way Anova test was conducted, a normality test was conducted with Shapiro Wilk. If the data is normally distributed, the One Way Anova test can be used in the analysis of this research data.

RESULTS AND DISCUSSION

Mean Value of Osteoblasts in Rat Femur Bones

Research was conductedin September to October 2024 with a sample size of 15 Wistar rats divided into 5 groups, each consisting of 3 Wistar rats.. Group 1 was given alcohol and normal vitamins.AndTreatment group 2given 20% (ethanol), ktreatment group 2given 250cc vitamin C and 1 ml 20% alcohol, kTreatment group 3 was given 500cc of vitamin C

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and 1ml of 20% alcohol and treatment group 4 was given 750cc of vitamin C and 1ml of 20% alcohol.

On the 14th day, the mice will be sacrificed and the femur bone tissue will be taken. The next process is making histology with dyes.*homoematoxylin-eosin*(HE). Histological observation was performed using a Zeiss Primo Star microscope in 3 large fields of view with 10 cal ocular magnification and 400x objective lens. Each preparation was observed and the average number of osteoblasts was calculated. The following are the results of descriptive statistics on the number of osteoblast cells.

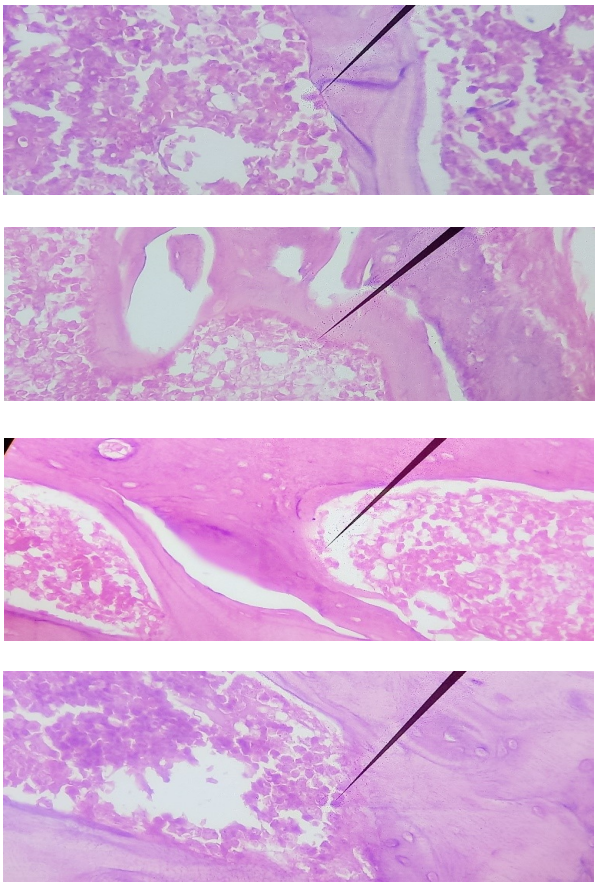

Figure 4. Osteoblasts (indicated by black arrows) in a section of the femur of a Wistar rat on day 14 with HE staining and 400x magnification.

Table 3. Descriptive Statistics of Osteoblast Cell Count

Group	Min	Max	Mean	St. Dev
Group of mice given alcohol and vitamin C (normal) (T1)	15	28	19.33	7,506
Group of mice given 1 ml of 20% alcohol (T2)	20	25	23.33	2,887
Group of mice given 250cc of vitamin C and 1 ml of 20% alcohol (T3)	10	37	26.33	14,364
Group of mice given 500cc of Vitamin C and 1 ml of 20% Alcohol (T4)	18	33	24.67	7,638

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Group	Min	Max	Mean	St. Dev
Group of mice given 750cc of vitamin C and 1 ml of 20% alcohol (T5)	7	11	9.33	2,082

Table 3 shows that the average value of osteoblasts in the group of mice given alcohol and vitamin C (normal) was 19.33 with a St.dev value of 7.506. The average value of osteoblasts in the group of mice given 1 ml of 20% alcohol was 23.33 with a St.dev value of 2.887. The average value of osteoblasts in the group of mice given vitamin C 250ccand 1 ml 20% alcohol of 26.33 with a St.dev value of 14.364. The average value of osteoblasts in the group of mice given 500 mg of vitamin Cccand 1 ml 20% alcohol of 24.67 with a St.dev value of 7.638. The average value of osteoblasts in the group of mice given vitamin C 7250ccand 1 ml of 20% alcohol of 9.33 with a St.dev value of 2.082.

Data analysis

The data obtained were then analyzed using a statistical analysis program on a computer with a significance level of 0.05 (p=0.05) and a confidence level of 95% (α = 0.05). The normality testing method used in this study is*Shapiro Wilk*with normal provisions if the significance > 0.05 to determine whether the data distribution is normally distributed or not. The results of the normality test are as follows:

Table 4. Data Normality Test with Shapiro Wilk

Variables	P	Information
Number of osteoblasts	0.607	Normally distributed

Based on the results of normality testing*Shapiro Wilk*a significance value of 0.607 was obtained (significance > 0.05), so it can be seen that the data is normally distributed, so that testing can be continued with a homogeneity test. The data obtained varied in each group. To find out how varied the data obtained was, a test was used.*Levene's test*with the provision of homogeneous if p > 0.05. The results obtained are as follows:

Table 5. Homogeneity Test

Variables	Levene's Value	P
Osteoblast	4,259	0.029

Table 5 shows that the results of the homogeneity test analysis obtained a Levene statistical value of 4.259, with a p value = 0.029 < 0.05, meaning that the average osteoblasts from the five groups did not come from the same data variance.

One Way ANOVA Test

From the average results it is known that there is an increase in the number of osteoblast cells. To prove whether the administration of vitamin C and alcohol gives different results on average for osteoblast cells, it can be seen from the test results.*One Way ANOVA*.

Table 6. Welch's Anova Test

Variables	P
Osteoblast	0.018

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Table 6 shows that the results of the Welch ANOVA test analysis obtained a p value = 0.018 < 0.05, meaning that at least there was a significant difference in the average osteoblasts in both groups.

Post Hoc Analysis

This test was conducted as a follow-up test to ANOVA which was conducted to see which sizes had differences in the number of osteoblast cells.

Table 7. Post Hoc Analysis

Group	Vitamin C Administration	P
Group T1	Group T2	0.562
	Group T3	0.319
	Group T4	0.443
	Group T5	0.165
Group T2	Group T1	0.562
	Group T3	0.662
	Group T4	0.846
	Group T5	0.062
Group T3	Group T1	0.319
	Group T2	0.662
	Group T4	0.808
	Group T5	0.029
Group T4	Group T1	0.443
	Group T2	0.846
	Group T3	0.808
	Group T5	0.044
Group T5	Group T1	0.165
	Group T2	0.062
	Group T3	0.029
	Group T4	0.044

Table 7 shows that there are several groups that have differences.mean osteoblast value. Statistically, there was a difference in the mean osteoblast between groups T3 and T5 with a p value = 0.029 < 0.05. There was a difference in the mean osteoblast between groups T4 and T5 with a p value = 0.044 < 0.05. From the research results table inCendikia Prospecta Medan Laboratory from September to October 2024, with 15 rat samples from 5 treatment groups, the average osteoblast value in the group of rats given alcohol and vitamin C (normal) was 19.33 with a St.dev value of 7.506. The average osteoblast value in the group of rats given 1 ml of 20% alcohol was 23.33 with a St.dev value of 2.887. The average osteoblast value in the group of rats given vitamin C 250ccand 1 ml 20% alcohol of 26.33 with a St.dev value of 14.364. The average value of osteoblasts in the group of mice given 500 mg of vitamin Cccand 1 ml 20% alcohol of 24.67 with a St.dev value of 7.638. The average value of osteoblasts in the group of mice given vitamin C 7250ccand 1 ml of 20% alcohol of 9.33 with a St.dev value of 2.082.

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According to Sintha Amelia Sari's research (2012), giving vitamin C is effective on osteoblast activity with an increase in the number of osteoblasts after extraction in male Wistar rats. Based on one-way ANOVA analysis, there was a significant difference on the 14th day. The results of the LSD test showed that there was no significant difference between groups K and P3 and groups P2 and P3 where p>0.05 while between the other groups there was a significant difference.

Based on the test resultsone way ANOVAobtained p value = 0.018 <0.05 means that at least, there is a significant difference in the average osteoblast in both groups. Then continued with the Post Hoc test which showed that there were several groups that had differences in the average osteoblast value. Statistically there was a difference in the average osteoblast between groups T3 and T5 with a p value = 0.029 <0.05. There was a difference in the average osteoblast between groups T4 and T5 with a p value = 0.044 <0.05.

According to researchers, this finding is in line with existing theories thatVitamin C has the potential to enhance the fracture healing process by increasing callus formation. Radiological and histological improvements may indicate increased bone density and possible acceleration in connective tissue formation. The importance of vitamin C in the fracture healing process is also related to its role in stimulating mesechymal stem cells to transform into osteoblasts (Levani, 2021).

CONCLUSION.

Based on the results of research on the administration of vitamin C to heal the femur bones of mice from 15 samples conducted in September - October 2024 inCendikia Prospecta Medan Laboratory, it can be concluded that: Administration of vitamin C can influence the increase in the number of osteoblast cells in the bone healing process. Of the 5 treatment groups, the average osteoblast value in the group of mice given alcohol and vitamin C (normal) was 19.33, the group of mice given 1 ml of 20% alcohol was 23.33, the group of mice given 250% vitamin Cccand 1 ml of 20% alcohol of 26.33, the group of mice given 500 mg of vitamin Cccand 1 ml of 20% alcohol of 24.67, the group of mice given vitamin C 7250ccand 1 ml of 20% alcohol is 9.33. Based on the results of the one way ANOVA test, the p value = 0.018 <0.05 means that at least, there is a significant difference in the average osteoblast in both groups. Then in the Post Hoc test there is a difference in the average osteoblast between groups T3 and T5 with a p value = 0.029 <0.05. There is a difference in the average osteoblast between groups T4 and T5 with a p value = 0.044 <0.05.

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