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Effect Of Hydroxyapatite Nanoparticle from Unam Snail (Volegalea Cochlidium) Shell on the Concentration of Alkaline Phosphatase in Osteoblast Cells (In Vitro)

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Abstract

One of the complications caused by periodontitis is bone loss; the treatment is bone grafting with bone graft material. Hydroxyapatite is a bioactive ceramic commonly used in bone healing and is the primary mineral present in mammalian bones and teeth. One source of HA powder is natural biological sources such as unam snail shells (*Volegalea cochlidium*). This research aimed to analyze the effect of hydroxyapatite nanoparticles from the Unam Snail (*Volegalea coccidium*) shell on the concentration of alkaline phosphatase in osteoblast cells in vitro. The fabrication of hydroxyapatite nanoparticles from Unam snail shells using a combination of mechanical-chemical methods known as ball mill and sol-gel. Osteoblast cells were isolated from rat calvaria and grown in a DMEM medium. Osteoblast cell differentiation was proven by examination of the alkaline phosphatase concentration and then reading it with an Elisa reader. The maximum average ALP concentration value was obtained at a concentration of 1.25 mg/mL on day 7, namely 70.38 ± 7.14 µL, then followed by the concentration of 2 mg/mL and control on the same day, namely 46.41 ± 3.10 µL and 44.51 ± 6.21 µL. The highest ALP concentration on days 1, 3 and 7 of all observation groups was obtained in the treatment group given Unam Snail (*Volegalea cochlidium*) shell hydroxyapatite nanoparticles at 1.25 mg/mL concentration. This study shows that administration of this material can increase the activity of differentiated osteoblast cells.

Keywords: hydroxyapatite; differentiation; osteoblast cells; unam snail shells

1. Introduction

Periodontal disease is a condition that might cause inflammation and destruction to the dental structure, namely the gingiva, periodontal ligament, cementum, and alveolar bone. The primary cause of periodontal disease is bacteria. The main etiology of periodontal disease is bacteria while predisposing factors include age, gender, knowledge, local factors, tooth brushing behavior, visits to the dentist, diet, and systemic factors.[1] The most common periodontal diseases that occur are gingivitis and periodontitis. Global Burden of Disease shows that periodontitis is the 6th most common disease globally, with 11.2% prevalence, and approximately 743 million individuals are impacted. Periodontal disease rose by 57.3% between 1990 to 2010.[2]

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Periodontitis is the leading cause of people losing teeth globally. Multiple tooth loss, edentulous, and masticatory dysfunction can all influence nutrition, quality of life, self-esteem, socioeconomic status, and healthcare expenses.[2] Periodontal treatment aims to maintain healthy teeth, periodontal tissue, peri-implant tissue, comfort, aesthetics, and function. Apart from that, other goals include the absence of recession, interproximal bone loss, and maximizing the function of dental implants.[3] One of the complications caused by periodontitis is bone loss; the treatment is bone grafting with bone graft material.[4]

Hydroxyapatite [Ca10(PO4)6(OH)2] is a biocompatible ceramic because it contains the same minerals as human teeth and bones. HA powder comes from two primary sources: chemically produced materials and natural biological sources such as snail shells, coral, egg shells, natural gypsum, natural calcite, and cow bones.[5]

Unam snails (formerly *Pugilina cochlidium*, now *Volegalea cochlidium*) are hard- shelled gastropods that thrive in Indonesian mangrove forest environments. *Volegalea cochlidium* is also found on substrates near beaches, muddy places, and estuaries. This snail is a sea mollusk that has long been recognized as a source of animal protein, high in calcium and important amino acids (arginine, leucine, and lysine). Still, Indonesians do not commonly use shells or snails. Precipitation, hydrothermal, mechanochemical, and sol-gel synthesis are all standard ways of producing hydroxyapatite. The sol-gel technique may create hydroxyapatite powder with a reasonably homogenous grain size, high degree of crystallinity, low processing temperature, and the capacity to make nanoparticles.[6,7,8]

Nanotechnology in hydroxyapatite has been confirmed to increase the biocompatibility and bioactivity of the material. In general, nanoparticles with various morphologies and sizes will have different properties, so nano-sized hydroxyapatite will show good morphology and crystallinity, proper stoichiometry, and high purity, and has many advantages. Therefore, much research has focused on examining the properties and advantages of synthesizing hydroxyapatite nanoparticles, especially those derived from natural materials, namely unam snail shells.[9]

Yan Chen et al., who studied the biocompatibility and differentiation of mouse MC3T3-E1 osteoblasts cultured on scaffolds injected with hydroxyapatite, chitosan, and collagen nanoparticles, it was found that the scaffolds injected with hydroxyapatite nanoparticles showed alkaline phosphatase (ALP), collagen type 1 (COL-1), RUNX- 2 and osteocalcin (OCN) which is a high osteogenic marker compared to other materials so it can be said that this material can facilitate osteoblast mineralization.[9]

Aini et al. discovered that unam snail shells could be used as a calcium source in the synthesis of hydroxyapatite from the shells of *Pugilina coccidium* and *Babylonia spir*it L. as a potential bone graft material for the application of periodontics.[6] Desy's research examined the administration of hydroxyapatite nanoparticles to 3T3 fibroblast cells in vitro. As a result, nanohydroxyapatite with concentrations of 0.8437 mg/mL and 1.6875 mg/mL did not have a toxic effect or had good bioviability properties against 3T3 fibroblast cells with a bioviability percentage above 90% for the group given 3.375 mg/mL nanohydroxyapatite had an effect, weakly toxic with a bioviability percentage above 78%. In comparison, the group that given 6.75 mg/mL nanohydroxyapatite had a strong toxic effect or very weak bioviability on fibroblast cells 3T3.[10]

Leni investigated the impact of unam snail shell hydroxyapatite nanoparticles (*Volegalea cochlidium*) at doses of 1.25 mg/mL, 1.5 mg/mL, 1.75 mg/mL, and 2 mg/mL on osteoblast cell survival in vitro. The study found that the group with a concentration of

1.25 mg/mL had the maximum osteoblast cell viability (164.60% ± 0.096), while the group with a concentration of 2 mg/mL had the lowest (74.23% ± 0.301). Leni concluded that the higher the dose of nHA administered, the fewer the surviving osteoblast cells. However, this work did not investigate the influence of unam snail shell hydroxyapatite nanoparticles on osteoblast differentiation activity and alkaline phosphatase concentration.[11] However, this research did not investigate the influence of unam snail shell hydroxyapatite nanoparticles on osteoblast differentiation activity and alkaline phosphatase concentration. According to the description above, researchers are interested in investigating the effect of supplying unam snail shell hydroxyapatite nanoparticles (*Volegalea cochlidium*) on the alkaline phosphatase concentration in osteoblast cells in vitro.

2. Material and methods

2.1. Types of research

This true experimental laboratory on osteoblast cell culture in vitro with a post test only control group design, namely by taking measurements after treatment. In this study, what was assessed was osteoblast cell differentiation as expressed by the concentration of alkaline phosphatase (ALP) after being treated with hydroxyapatite nanoparticles from unam snail shells (*Volegalea coccidium*) with different concentrations and duration of administration.

2.2. Research sites

The location for collecting samples of unam snail shells (*Volegalea cochlidium*) was carried out in the Serdang Bedagai Sea Area, North Sumatra. Unam snail shell (*Volegalea cochlidium*) hydroxyapatite nanoparticles were synthesized at the Phytochemistry Laboratory, Faculty of Mathematics and Natural Sciences, University of North Sumatra. Osteoblast cells (cell line) were obtained from the UPT Integrated Laboratory and Technology Innovation Center-CDAST, Jember University, and tested for ELISA Alkaline phosphatase (ALP) Assay for osteoblast cells after administration of unam snail shell hydroxyapatite nanoparticles (*Volegalea cochlidium*).

2.3. Hydroxyapatite Synthesis Procedure

The hydroxyapatite synthesis method used is the sol-gel method. A total of 8.52 grams of disodium hydrogen phosphate powder (Na2HPO4 98%) was dissolved in 100 ml of aquabides until it reached 0.6 M. Then add 100 ml of Ca(NO3)2 solution little by little using magnetic stirring for 30 minutes until the Ca/P ratio was reached. 1.67. After the mixture was formed, the mixture was stirred again for 15 minutes to trigger precursor reactivity. The solution was left for 24 hours at room temperature, then refluxed in an oil bath for 16 hours at 70°C and evaporated into a water bath for 15 hours at 100°C. The resulting gel was dried in an oven and smoothed using a mortar and pestle. The powder that has been ground is then sintered at a temperature of 800°C.

2.4. Osteoblast Cell Incubation Process

Osteoblast cell cultures in the form of cell lines were stored in roux bottles containing Dulbeco's Modified Eagle's Medium (DMEM) and 10% bovine serum until they were confluent for 4 days, then incubated in a standard incubator for 24 hours at 37oC. DMEM media and 10% bovine serum as growth media were removed from the roux bottle. Osteoblast cell cultures in roux bottles were released with 0.5% trypsin- versene for 20 minutes. The detached osteoblast cell culture was then given growth media containing 10% FBS. The cell suspension was centrifuged, and DMEM + 10% FBS was added to the cell precipitate. Cells were put into 96 well plates for cell culture, growth media, and growth media only, 100 µl each as many replicates as needed, and incubated in a 5% CO2 incubator at 37oC for 24 hours.

2.5. Osteoblast Cell Activity Testing: ELISA ALP Assay

This research carried out ALP tests on all treatment groups at concentrations of 2 mg/mL and 1.25 mg/mL, with each group treated for different durations, namely 1 day, 3 days and 7 days. The ALP test consists of the components of ELISA ALP Assay Buffer, nitrophenyl phosphate (pNPP), ALP Enzyme, Stop Solution.

2.6. Sample Preparation

ALP in the cell culture medium was measured directly without dilution. Cultivated cells can be homogeneous in an Assay Buffer to determine intracellular ALP and centrifuged at 13,000g for 3 minutes to remove insoluble

material. Fill the 96-well plate with samples of various volumes and add Assay Buffer until the volume reaches 80 µm. A 405 nm wavelength/optical density spectrophotometer may be used to read sample colors. Samples were reinserted into separate wells 80 µm apart. Add 20 µl of cease and mix thoroughly to inhibit the sample's ALP metabolism. Add 50 µl of 5 mM pNPP reagent to every well with the sample and control, and mix evenly. Place the sample for 60 minutes at 25ºC, away from light.

2.7. Standard Curve

Mix 40 µl of 5 mM pNPP reagent in 160 µl of Assay Buffer to make a standard solution of 1 mM pNPP. Then add 0, 4, 8, 12, 16, 20 µl into 96 well plates in 2 copies to make 0, 4, 8, 12, 16, 20 nmol/well pNPP standard. Add Assay Buffer until the solution volume becomes 120 µl. Mix 10 µl of ALP enzyme to every well containing the pNPP standard solution. ALP will convert the same amount of substrate into p-Netrophenol (pNP). Then, the sample was set in the incubator for an hour at 25ºC away from light. Cease all reactions by adding 20 µl of Stop solution to every standard solution and sample, excluding the control group that was not given, and vibrate the plate gently. Observe the optical density at a wavelength of 405 nm with a micro plate reader.

To prepare a standard solution of 1 mM pNPP, mix 40 µl of 5 mM pNPP reagent with 160 µl of Assay Buffer. Fill two 96-well plates with 0, 4, 8, 12, 16, and 20 µl each to provide a pNPP standard of 0, 4, 8, 12, 16, and 20 nmol/well. Add Assay Buffer until solution volume reaches 120 µl. Add 10 µl of ALP enzyme to all with the pNPP standard solution. ALP Enzyme will convert an equal quantity of pNPP substrate into p- Netrophenol (pNP). Incubate the sample for 60 minutes at 25oC, away from light. To stop all reactions, apply 20 µl of Stop solution to each standard solution and sample, except for the control group (which was not supplied), and shake the platter slightly. Observe the optical density at the wavelength of 405 nm with a micro plate reader.

2.8. Processing and analysis of data

The measured data of each variable are then calculated, tabulated, and processed computerized. Data from each examination is analyzed univariately by presenting the data in a table in the form of single data in the form of averages and standard deviations for ratio or numerical data. Next, the data was analyzed bivariate normality with the Shapiro-Wilk test, then continued with the T-Independent Test statistical test to see the differences intragroup that had been given simultaneous concentration and duration of treatment.

3. Results

This study used samples of osteoblast cells treated with nanohydroxyapatite from Unam snail shells with different concentrations. Each concentration group was divided into three groups based on different observation times, namely days 1, 3, and 7, and each group was replicated three times. Based on the results of observations in all groups, the upper average ALP concentration value was obtained at a concentration of 1.25 mg/mL on day 7, namely 70.38 ± 7.14 µL. In contrast, the lowest was obtained at 2 mg/mL concentration on the first day, namely 45.62 ± 1.26 µL (Table 1).

Based on the bar diagram, the average and standard deviation of ALP concentration show almost the same pattern in all groups from days 1 to 7, showing an increase in the average value of ALP concentration (Figure 3).

3.1. Analysis of average ALP concentrations across concentration differences groups on day 1

Based on the results of observations on the first day of application of nanohydroxyapatite from Unam snail shells, the highest average ALP concentration for each concentration group was obtained in the group of 1.25 mg/mL concentration was 54.51 ± 1.92 µL and the lowest was in the 2 mg/mL concentration group, namely 45.62 ± 1.26 µL. The statistical test results for the entire group showed a value of $p=0.003$ ($p<0.05$), which means that

statistically, there was a significant difference in the average value and standard deviation of the entire group on the first day of observation (Table 2).

Table 1. Average ALP concentration in all study groups

	Concentration	Day	ALP concentration (µL)
1.	1,25 mg/mL	1	54,51 ± 1,92
		3	58,48 ± 1,72
		7	70,38 ± 7,14
2.	2 mg/mL	1	45,62 ± 1,26
		3	46,10 ± 2,18
		7	46,41 ± 3,10

Table 2. Difference in average ALP concentration of all groups on day 1

No	Sample Group	Mean ± SD ALP concentration (µL)	p-value
1	1,25 mg/mL	54,51 ± 1,92	0.003*
2	2 mg/mL	45,62 ± 1,26	

*Independent T Test statistical test, significance level p<0.05

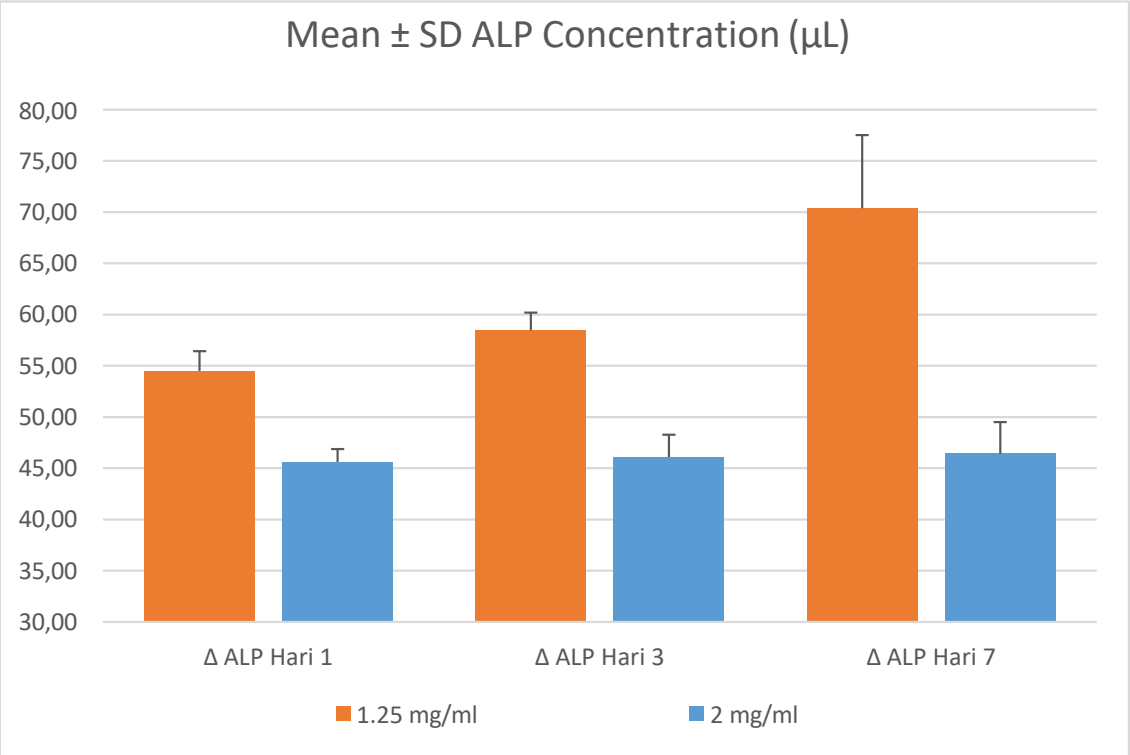

Figure 3. Bar diagram of the average and standard deviation of ALP concentrations for all groups on days 1, 3, and 7.

3.2. Analysis of average ALP concentrations across concentration difference groups on day 3

The results of observations on the third day of application of nanohydroxyapatite of Unam snail shells, the highest average ALP concentration of each concentration group and control was also shown by the 1.25 mg/mL concentration group of 58.48 ± 1.72 µL and the lowest was in the 2 mg/mL group. mL, namely 46.10 ± 2.18 µL. The statistical test results for the entire group showed a value of p=0.002 (p<0.05), which means that statistically, there was a significant difference in the average score and standard deviation of the entire group on the third day of observation (Table 3).

Table 3. Difference in average ALP concentration of all groups on day 3

No	Sample Group	Mean ± SD ALP concentration (µL)	p-value
1	1,25 mg/mL	58,48 ± 1,72	0.002*
2	2 mg/mL	46,10 ± 2,18	

*Independent T Test statistical test, significance level p<0.05

3.3. Analysis of differences in average ALP concentrations across concentration groups on day 7

The following results show observations on the seventh day of application of Unam snail shell nanohydroxyapatite. The highest average ALP concentration of each concentration group and control was also shown by the 1.25 mg/mL concentration group of 70.38 ± 7.14 µL and the lowest in the 2 mg/mL, namely 46.41 ± 3.10 µL. The statistical test results for the entire group showed a value of p=0.006 (p<0.05), which means that statistically, there was a significant difference in the average value and standard deviation of the entire group on the seventh day of observation (Table 4).

Table 4. The difference in mean ALP concentration of all groups on day 7

No	Sample Group	Mean ± SD ALP concentration (µL)	p-value
1	1,25 mg/mL	70,38 ± 7,14	0.006*
2	2 mg/mL	46,41 ± 3,10	

*Independent T Test statistical test, significance level p<0.05

3.4. Analysis of differences in average ALP concentrations between days 1, 3, and 7 at a concentration of 1.25 mg/mL

Observation results from the group that applied nanohydroxyapatite snail shells at a concentration of 1.25 mg/mL on days 1, 3, and 7, the highest average ALP concentration results were obtained on the seventh observation day, namely 70.38 ± 7.14 µL and shows an increasing pattern from the beginning to the end of the observation. The statistical tests in the groups show a value of p=0.011 (p<0.05), which means a statistically significant difference in the average value and SD of ALP concentrations from all days of observation in the 1.25 mg/mL concentration group (Table 5).

The results of the follow-up test (Post hoc) obtained a comparison of observation days, which gave a significant difference (p<0.05) in the 1.25 mg/mL concentration group, namely between observation days 1 and 3 and day 7, but between days 1st and 3rd do not show any significant differences (Table 6)

Table 5. The difference in average ALP concentration between days 1, 3, and 7 at a concentration of 1.25 mg/mL

No	Sample Group	Mean ± SD ALP concentration (µL)	p-value
1	Day 1	54,51 ± 1,92	
2	Day 2	58,48 ± 1,72	0.011*
3	Day 3	70,38 ± 7,14	

*ANOVA statistical test, significance level p<0.05

Table 6. Follow-up test (Post hoc) difference in average ALP concentration between groups at a concentration of 1.25 mg/mL

No	Sample Group		p-value
1	Day 1	Day 7	0,310
2		Day 7	0,004*
3	Day 3	Day 7	0,016*

*LSD test, significant score p<0.05

3.5. Analysis of differences in average ALP concentrations between days 1, 3, and 7 at a concentration of 2 mg/mL

Observation results from the group that applied snail shell nanohydroxyapatite at a concentration of 2 mg/mL on days 1, 3, and 7, the highest average ALP concentration was also obtained on the seventh observation day, which was slightly different from the third day, namely 46.41 ± 3.10 µL and also showed an increasing pattern from the beginning to the end of the observation. The results of statistical tests in the groups also show a value of p=0.915 (p>0.05), which means that statistically, there is no significant difference in the average value and standard deviation of ALP concentrations from all days of observation in the 2 mg/mL concentration group (Table 7).

Table 7. Difference in average ALP concentration between days 1, 3 and 7 at a concentration of 2 mg/mL

No	Sample Group	Mean ± SD ALP concentration (µL)	p-value
1	Day 1	45,62 ± 1,26	
2	Day 3	46,10 ± 2,18	0.915*
3	Day 7	46,41 ± 3,10	

*ANOVA statistical test, significance level p<0.05

4. Discussion

Osteoblast cell activity, particularly during the differentiation stage, is commonly utilized to assess the influence of biomaterials on biological responses. Differentiated osteoblast cells produce type I collagen, alkaline phosphatase, and non-collagenous extracellular bone matrix proteins such as osteonectin, osteocalcin, osteopontin, and bone sialoprotein. This bone matrix protein has been demonstrated to be beneficial as a bone formation marker.[12,13] Therefore, this study employed Alkaline Phosphatase (ALP) as a osteoblast cell differentiation activity marker. Alkaline phosphatase (ALP) is regarded as a precursor of osteoblast differentiation; increased ALP levels indicate active bone development because ALP is a byproduct of osteoblast activity; and tissue-specific ALP levels are regarded as a possible indicator of cells that differentiate according to the mineralization phenotype.[14]

Statistical results showed significant differences between the two groups on days 1, 3, and 7. These results obedient to Leni A. et al.'s data, who examined the same material on the bioviability of osteoblast cells, stated that the optimal concentration for giving nanohydroxyapatite from Unam snail shells was at a low concentration and concluded that the maximum concentration of nanohydroxyapatite given, the lower the cell viability observed.[11]

Similar to this research on the first day of observation, the lowest concentration shows the highest ALP concentration compared to the group with a higher concentration, and the longer the observation time, the clearer the difference between nanohydroxyapatite concentration 1.25 mg/mL and the 2 mg/mL concentration group, this is because according to previous research, this is the optimal concentration for cells to grow. In accordance with Wang R. et al.'s research, which intended to examine the influence of nano-HAP on osteoblast development, MC3T3E1 cells were tended with variant doses of nano-HAP, then ALP activity was determined on the seventh day after treatment. The results showed an increase in ALP activity depending on the dose when the concentration was ≤100 μg/ml, but there was no significant increase when the concentration reached 200 μg/ml.[15] Furthermore, Wrobel E. et al. discovered a significant relation in the viability of human bone-derived cells and ALP concentration, as well as the fact that these cells were very active in proliferation and differentiation in the first seven days of observation.[13]

The high viability in the treatment group, especially at the low concentration, namely 1.25 mg/mL, was caused by the nanohydroxyapatite content of the Unam snail shell (*Volegalea cochlidium*) used in this study, which is rich in calcium. This calcium content is a second messenger that functions as a cellular receptor mediator for cell proliferation, movement, secretion, and neotransmission but also has a role in the occurrence of apoptosis physiologically and pathologically, including in osteoblast cells; calcium can also act as a regulator of apoptosis by modulating phosphate ions to activate the phosphate-mediated autoblast apoptosis pathway.[16] In addition, research by Forget

F. et al. stated that high concentrations of extracellular calcium could also induce cell apoptosis. Apart from the content, the size of the hydroxyapatite particles used also has an essential function in maximizing the activity of osteoblasts for differentiation.[17] Liu's 2012 research comparing the efficiency of nanohydroxyapatite with various sizes and crystallization on osteoblast cells showed a significant difference in ALP activity in cells treated with nHA compared to those that tended with nHA control cells.[12] According to Wang R. et al. This is because nanoparticles have high surface energy; they always aggregate at higher concentrations in the medium.[15]

Even though the presence of Ca2+ ions in hydroxyapatite is in a bound state, the presence of Ca2+ ions comes from the nature of the hydroxyapatite material itself. The ability to release Ca2+ ions of nanohydroxyapatite in this study, apart from being based on the dissolved properties of the material, is also due to the nano-sized size of the hydroxyapatite particles. Previous studies have stated that apatite particles measuring 20- 40 nm play a crucial role in osteogenesis biominerals, and they suggest that the basic inorganic material of teeth and bones may be nano-sized apatite particles. The lack of nano blocks is in a collagen matrix and then interacts with one another to produce a biomaterial with chemical and physical qualities, such as toughness, development insensitivity/dissolving ability, and adaptable form.[18] The dissolving ability of nanohydroxyapatite has been researched and proven by Mocanu A et al., which examined the release of ions from nanohydroxyapatite and substituted HAP (ms-HAP) in various soakings in vitro; it was found that there was an increase in the release of various ions studied, one of which was Ca2+ along with the length of soaking of all hydroxyapatite samples, apart from that, this study also concluded that the observed ion release kinetics proves that ion release is largely based on the diffusion process.[19] Then, further research was conducted by Szalaj U. et al. Regarding the increase in the delivery of Ca2+ ions from nanohydroxyapatite by increasing the specific facet area and particle size in vitro, it was concluded that the smaller the particle dimension and the specific facet of the hydroxyapatite, the higher the release of Ca2+ ions.[20] The influence of particle dimension on particle dispersity and bioviability has widely researched. Reducing particle size - in this case, nanohydroxyapatite - aims to reduce the thickness of the diffusion surface around the particle, thereby increasing the concentration gradient and degradation speed of this material.[21]

There is a lot of research on nanohydroxyapatite and its osteoinduction effects. Most studies show that hydroxyapatite accelerates osteoblast differentiation in vitro. This is proven by the data of the research; all groups showed an increase in the average ALP concentration from days 1, 3, and 7. However, only the concentration of 1.25 mg/mL showed a significant elevation between days of observation, namely sequentially from 54.51 ± 1.92 μL, 58.48 ± 1.72 μL and 70.38 ± 7.14 μL. Based on follow-up tests, the average ALP concentration between days 1

and 3 showed no difference; this is the same as a study by Chen Y et al., which examined the differentiation of MC3T3-E1 osteoblast cells with a combination of chitosan-nanohydroxyapatite and with chitosan alone and then assessed by measuring ALP activity after 1, 3, 7, and 10 days of cultivation. The results of changes in ALP pursuit for cells cultured on the CS/nHAC surface compared to CS revealed that the ALP activity of MC3T3-E1 cells extends in both groups wasn't significantly different on days 1 and 3, but MC3T3-E1 cells cultured on the CS/nHAC scaffold had much higher ALP activity than cells cultured in the CS group on days 7 and 10. The ALP activity in the CS/nHAC group peaked on day 7. The reduction on day 10 was most likely caused by the cell culture progressing to an advanced stage.[9]

This increase in average concentration can be caused not only by this concentration being the ideal concentration for osteoblast cell viability but also by the process of cell autophagy, which is induced by the content, concentration, and particle size of Unam snail shell (*Volegalea coccidium*) nanohydroxyapatite particles. Autophagy is a biological mechanism that recycles cellular components while maintaining cell homeostasis. Several proteins related to autophagy activity are necessary for the growth, survival, and activity of bone cells, including osteoblasts.[24,25]

Research conducted by Wang R. et al., who observed the administration of different concentrations of nanohydroxyapatite to modulate differentiation through the initiation of autophagy by the mTOR signal pathway, showed that nanohydroxyapatite could modulate the differentiation of MC3T3E1 osteoblast cells through the induction of cell autophagy depending on the concentration of administration. Lower concentrations of nanohydroxyapatite showed moderate cell autophagy and promoted osteoblast differentiation, while higher concentrations of nano-HAP induced excessive cell autophagy and had no effect or inhibited osteoblast differentiation. For the first time, the study showed that nanohydroxyapatite can trigger autophagy in osteoblasts depending on the dose.[15] The research conducted by Ha SW et al., who researched the administration of nanoparticle-sized bioactive silica materials to assess the increase in osteoblast differentiation through the autophagy process, stated that nanoparticle-sized bioactive materials were proven to stimulate the autophagy process of osteoblast cells through the MAPK ERK1/2 pathway.[26] Wang J et al., in their literature review, stated that it has been proven that calcium functions in autophagy through the MTOR and AMPK signaling pathways; in addition, cytosolic calcium content is closely related to the regulation of the autophagy process in osteoblast cells. Cytosolic calcium influences not only the early stages of autophagy but also the proximal and distal stages through the autophagy process. Calcium and phosphorus are important ionic components for the genesis of hydroxyapatite during bone extracellular matrix mineralization, and defects in the autophagy process can reduce mineralization capacity.[24]

5. Conclusion

Unam Snail (*Volegalea coccidium*) shell hydroxyapatite nanoparticles with a 1.25 mg/mL concentration were the optimum dose with the highest average ALP concentration value on days 1, 3, and 7 of all observation groups, namely 54.51 ± 1.92 µL; 58.48 ± 1.72 µL and 70.38 ± 7.14 µL.

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