

ORIGINAL ARTICLE

The Effect of Hydroxyapatite-Gelatin-Propolis Composite on the Viability, Proliferation, and Mineralization of Osteoblast Cells (*In Vitro*)

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ABSTRACT

Introduction: Hydroxyapatite (HA) and gelatin and propolis materials have been investigated and combined to enhance bone regeneration. This study aimed to evaluate the levels of total protein, protein profile, alkaline phosphatase (ALP) and osteocalcin (OC) in the culture medium of osteoblast cells following exposure to HA, gelatin, and propolis elution. **Methods:** Human Osteoblast Cell-line MG-63 was cultured in six exposure groups, including control, HA, 6% propolis, HA-gelatin, HA-6% propolis, and HA-gelatin-6% propolis. ALP and OC levels were analyzed and quantified in the culture medium after 7, 14, and 21 days of exposure using the ELISA test. Then the Bradford test was performed to see the total protein and SDS-PAGE test to see the protein profile. **Results:** Higher levels of total protein were observed in the HA group, HA-6% propolis group, and the 6% propolis group compared to the control group. Conversely, no differences were noted in the protein profiles among all exposure groups. The HA group, 6% propolis, and HA-gelatin exhibited higher levels of ALP and OC levels compared to the control group. ALP levels were increase positively correlated with an increase in OC levels in all group. **Conclusions:** There was no significant difference in the total protein, protein profile, levels of alkaline phosphatase and osteocalcin in the hydroxyapatite-gelatin-propolis 6% elution exposure group compared to the control group. However, the results of the study showed that there was an increase in the total protein, ALP level, and OC level of the osteoblast cell culture following the administration propolis.

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INTRODUCTION

Bone is mineralized connective tissue, plays a pivotal role in our body, providing support, protection to soft tissue, and serving as a reservoir for calcium, phosphate, and bone marrow (1). Compared to other tissues, bone possesses remarkable self-healing abilities (2, 3), maintaining homeostasis through a dynamic balance of resorption and regeneration (3). However, spontaneous bone regeneration is limited, particularly in cases of extensive defects caused by trauma, tumors, infections, or congenital abnormalities (4,5) leading to functional deficits and impacting the patient's quality of life.

Various bone graft materials exist, including autografts,

allografts, xenografts, and alloplasts (6). Autografts, although histocompatible, have drawbacks such as donor site morbidity and limited availability (7). Allografts and xenografts offer alternatives but pose risks of immune rejection and disease transmission (7). Recognizing these limitations, bone tissue engineering emerged as an alternative approach (5, 8).

Scaffolds are integral to bone tissue engineering, providing structural support and a conducive environment for cell proliferation and differentiation (9). Common scaffold materials include inorganic compounds and natural or synthetic polymers (10).

Hydroxyapatite (HA) is the main natural inorganic component of bone that exhibits excellent biocompatibility, bioactivity, osteoconductivity, non-toxicity and non-inflammatory characteristics. However, HA has slow biodegradation and poor mechanical properties, such as brittleness, low fatigue resistance,

and low strength and endurance (11).

Gelatine is a collagen-derived polymer and is known as a biocompatible and biodegradable material (12). Gelatine is prominently used in the pharmaceutical and medical fields, such as drug delivery carriers, tissue engineering scaffolds, and wound dressing materials (13). Gelatine can be made in a sponge form for suitability in tissue engineering applications. The porous 3D structure of the gelatine sponge scaffold can give ample space for cell adhesion. Nevertheless, gelatine scaffolds suffer from hydrolysis susceptibility and inadequate mechanical properties (13).

Another ingredient that has been widely used in the world of health is propolis, because it has anti-proliferative, anti-inflammatory, antioxidant, antimicrobial properties. Propolis induces wound healing by promoting wound epithelialization. Propolis and propolis-derived compounds have been proven to hinder osteoclastogenesis and bone resorption by regulating the receptor activator of nuclear factor kappa-B ligand (RANKL) signalling pathway, which has the potential to be useful in the treatment of bone resorption associated with traumatic dental injuries (14). To develop new materials, a medical material must be tested to determine its biocompatibility when in contact with the body (15). Because the new material to be developed is intended for bone tissue engineering, the material's biocompatibility with bone cells needs to be known. One of the crucial indicators in biological evaluation research is the cytotoxicity test using tissue cells in vitro to observe the effect of a medical device or material on cell growth, reproduction and morphology (15, 16). The MTT test is the 'gold standard' colorimetric test in assessing viability and proliferation cells through measuring cell metabolic activity (17, 18).

MATERIALS AND METHODS

This study was designed as an in vitro experiment. Cells used were osteoblast-like MG-63 cells. Cells were cultured up to near-confluence in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and 3% antibiotic-antifungal, at 37 °C with 5% CO₂ in air. The culture medium was replaced every 3 days. Cells were then harvested and seeded at 1 × 10⁵ cells per well, followed by an overnight incubation to facilitate cell adhesion. The cells were then divided into two groups, a treatment group with media containing elution with various concentration of hydroxyapatite, gelatin and propolis, and a negative control group, incubated only with the media.

Hydroxyapatite (BATAN), Gelatine (Merck), and Propolis from Trigona Sp were made into elution with 7.5%w/v solid content. The weight ratio of apatite to

solid content was 30%w/w, while two distinct propolis concentrations, namely 6% and 10%, were used, followed by gelatin. To create the elution, the following steps were followed: Gelatin was dissolved in saline in a beaker glass heated on a hot plate at 40 °C while stirring using a glass rod stirrer. HA and UI propolis are then added according to the specified concentration. The mixture of ingredients was put into a 15 ml tube and mixed further using a vortex. The tube was centrifuged for 5 minutes, 2000 g. Filter the elution with a 20-25 micron microfilter before placing it in a 15 ml tube.

To estimate the density of viable cells, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted on day 1 and 8 after exposure. The samples were divided into 1 × 10⁵ cells/well in a 96 well plate. The treatment groups received a) hydroxyapatite, b) propolis 6%, c) propolis 10%, d) hydroxyapatite-propolis 6% (HA-P 6%), e) hydroxyapatite-propolis 10% (HA-P 10%), f) hydroxyapatite-gelatin, g) hydroxyapatite-gelatin-propolis 6% (HA-GEL-P 6%), and h) hydroxyapatite-gelatin-propolis 10% (HA-GEL-P 10%). Each experiment was performed in 6 replicates. Control group and treatment groups that have been incubated for 1 day and 8 days were examined with a microscope to identify cell seeding errors and cell growth characteristics. Cells were observed using an inverted microscope with 10x magnification. MTT solution, prepared by combining 5 grams of MTT powder with 1 ml of saline, was added to each well and incubated for 2 hours. Afterwards, the solution was discarded and 100 µL of Acidified Isopropanol solution was added to each well. The plate was subsequently wrapped with aluminum foil and shaken on an orbital shaker for one hour at room temperature. The absorbance was measured using a microplate reader at wavelength of 600 nm. Data analysis was preceded by a normality test using the Shapiro-Wilk test. The results were statistically analyzed by Mann Whitney for abnormal data. P value of < 0.05 represented a significant difference.

To assess proliferation and mineralization, Alizarin Red Staining (ARS) assays were conducted on the treatment groups at various time intervals, specifically on day 7, 14, and 21. The samples were divided into 1 × 10⁵ cells/well in a 24 well plate. Each experiment was performed in 3 replicates. The culture medium was removed, cells were washed with PBS at 37 °C, and the attached cells were fixed in 10% formalin for 24 hours at room temperature. Cells were stained with Alizarin Red (Alizarin Red S) and shaken for 15 minutes at room temperature. Afterward, the Alizarin Red was eliminated and the specimens were washed with sterile water to eliminate any surplus color. Cells subsequently were examined under microscope. Quantitative analysis of calcium deposition and osteoblast cell counts was conducted using ImageJ software.

RESULT

Figure 1 showed cell viability on day 1 and 8 after exposure. The cell viability of 6% propolis and hydroxyapatite groups were more than 100% on day 1. The 10% propolis, 6% hydroxyapatite-propolis, and 6% hydroxyapatite-gelatine-propolis groups showed cell viability of less than 100% but more than 70%. When compared with the negative control group, the cell viability of the 10% propolis group was 29.33% lower, the 6% hydroxyapatite-propolis group was 27.54% lower, and the 6% hydroxyapatite-gelatine-propolis group was 7.2% lower. The lowest cell viability on day 1 was shown by the 10% hydroxyapatite-gelatine-propolis group with a percentage 54.66% less than the negative control group. Nevertheless, there was a notable standard deviation observed in the hydroxyapatite, 10% hydroxyapatite-propolis, 6% hydroxyapatite-gelatine-propolis, and 10% hydroxyapatite-gelatine-propolis group. All groups showed cell viability below 50% on day 8.

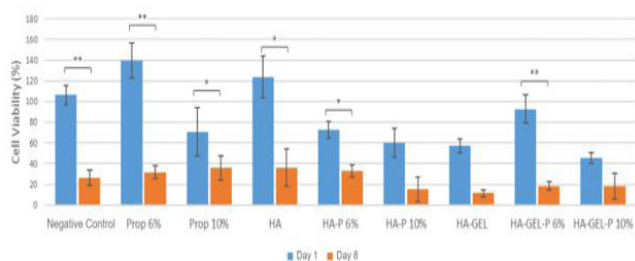


Figure 1: Comparison of cell availability upon treatment at day 1 and 8

Figure 2, showed cell only on days 7, 14, and 21 there is data from samples that have values that are very different from the sample average on those days. Examples on days 7 and 14 are HA-Prop6% and on day 21 HA-Prop6% and HA-Gel-Prop6%.

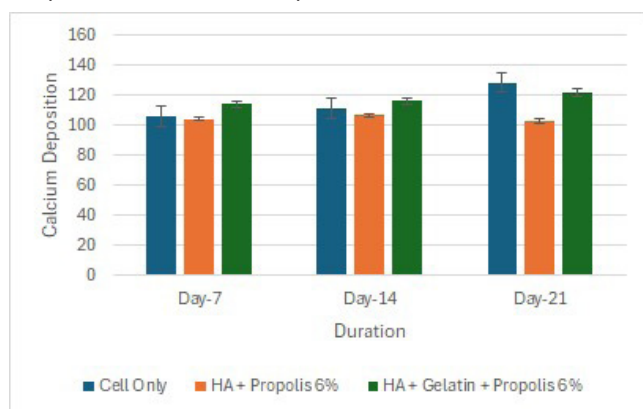


Figure 2: Cell amount on day 7, 14, 21

Following Figure 3 the statistical analysis, calcium deposition was observed. On day 7, the cell only group had a mean of 106.18 (95% CI: 100.19-112.16), while the hydroxyapatite-propolis 6% group had a mean of 104.16 (95% CI: 84.37 - 123.95), and the hydroxyapatite-gelatine-propolis 6% group had a

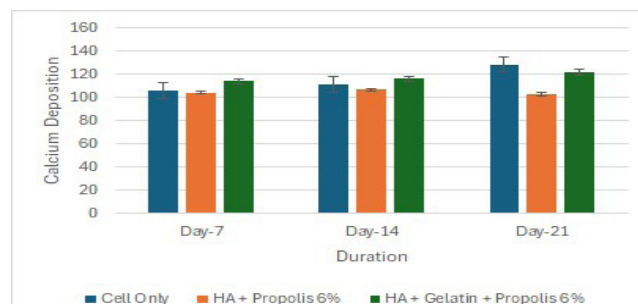


Figure 3: Calcium deposition on day 7, 14, 21

mean of 114.03 (95% CI 103.76-124.30). The one-way ANOVA test yielded a p-value of 0.136 (>0.05), indicating no significant differences among groups. On day 14, the cell only group showed a mean of 111.64 (95% CI: 101.92-121.36), the hydroxyapatite-propolis 6% group had a mean of 106.81 (95% CI: 96.38 - 117.23), and the hydroxyapatite-gelatine-propolis 6% group had a mean of 116.09 (95% CI 110.99-121.19). The one-way ANOVA test resulted in a p-value of 0.059 (>0.05), indicating no significant differences. By day 21, the cell only group exhibited a mean of 128.56 (95% CI: 110.80-146.32), the hydroxyapatite-propolis 6% group had a mean of 102.79 (95% CI: 90.41-115.17), and the hydroxyapatite-gelatine-propolis 6% group had a mean of 121.97 (95% CI: 113.79-130.15). The one-way ANOVA test yielded a p-value of 0.003 (<0.05), indicating a significant difference. Post hoc tests revealed significant differences between cell only and hydroxyapatite-propolis 6% on day 21 ($p = 0.003$) and between hydroxyapatite-propolis 6% and hydroxyapatite-gelatine-propolis 6% on day 21 ($p = 0.014$).

Regarding cell counts in the cell only group, on day 7, the average was 2135.33 (95% CI: 1810.94 - 2459.72), on day 14, it was 2274.00 (95% CI: 2026.84 - 2485.15), and on day 21, it was 1860.00 (95% CI: 1659.47 - 2060.52). The one-way ANOVA test resulted in a p-value of 0.007 (<0.05), indicating a significant difference. Post hoc tests revealed significant differences between day 7 and day 21 ($p = 0.048$) and between day 14 and day 21 ($p = 0.007$).

In the hydroxyapatite-propolis 6% Group, on day 7, the average was 1348.00 (95% CI: 1099.67 - 1596.32), on day 14, it was 4154.33 (95% CI: 3844.96 - 4463.69), and on day 21, it was 1248.33 (95% CI: 1053.27 - 1463.39). The one-way ANOVA test yielded a p-value of 0.000 (<0.05), indicating a significant difference. Post hoc tests revealed significant differences between day 7 and day 14 ($p = 0.000$) and between day 14 and day 21 ($p = 0.000$).

For the hydroxyapatite-gelatine-propolis 6% Group, on day 7, the average was 2064.66 (95% CI: 1679.95 - 2449.38), on day 14, it was 1727.00 (95% CI: 1420.02 - 2033.97), and on day 21, it was 3444.00 (95% CI: 3161.51 - 3726.48). The one-way ANOVA test yielded

a p-value of 0.000 (<0.05), indicating a significant difference. Post hoc tests revealed significant differences between day 7 and day 21 ($p = 0.000$) and between day 14 and day 21 ($p = 0.000$).

DISCUSSION

Research using in vitro culture allows for direct assessment of the response of osteoblast cells to the materials tested (19). In this study, researchers used the MG-63 osteoblastic cell line to evaluate the effect of propolis-hydroxyapatite-gelatine on cell viability. MG-63 cells are an osteoblastic model that shows the functional characteristics of pre-osteoblastic stage cells (20-21). Compared with human primary osteoblasts, MG-63 cells have several advantages, namely, ease of maintenance in many culture passages, unlimited number of cells, and a different phenotype. relatively stable (21). These cells are commonly used in research on bone cells (20). In this study, cells were sub cultured using DMEM media supplemented with FBS and antimycotic antibiotics. The cell medium was changed every three days to meet the nutritional needs of the cells (22). Cell growth in this study was observed using an inverted microscope with 10x magnification. Cells show varying shapes ranging from oval to spindle. On day 1 after exposure, the cell morphology of each treatment group was relatively similar to that of the control group. On day 8 after exposure, the cells appeared thin and irregular in shape.

The combination of hydroxyapatite-gelatine and propolis was made in elution form by adjusting the composition of the ingredients using the method of Sunarso et al who reported that the ratio of 30%w/w hydroxyapatite to 7.5%w/v in 100 ml of water for making hydroxyapatite-gelatine showed compressive strength properties. which compares favourably with other compositions containing less gelatine. Compressive strength is an important aspect in bone tissue engineering so that biomaterials can be implanted and substitute for the physiological function of human bone (23).

Hydroxyapatite has long been used in regenerative science and is a popular bio ceramic in bone tissue engineering due to its chemical structure that is very similar to apatite carbonate which is the main inorganic component of bone. Meanwhile, gelatine is a natural polymer that shows a biomaterial function similar to collagen in cellular development but with a lower level of degradation and with non-immunogenic by-products (12, 24). Gelatin-hydroxyapatite composites have been developed for use as scaffolds for bone regeneration. With a combination of two or more components with different physicochemical properties, the usefulness and advantages of scaffolds in terms of biological effects are expected to be superior to scaffolds with one component (25).

In this research, hydroxyapatite and gelatine were added with propolis. The propolis concentrations used in this research were 6% and 10%. Sutjarittangtham et al reported that at a concentration of 6%, the fibres in propolis were still elastic, while at higher concentrations, the fibres became brittle. Apart from that, a propolis concentration of 2-8% is also reported to have a good bactericidal effect (26). Research by Soekanto et al developed a mixture of Casein Phosphopeptide–Amorphous Calcium Phosphate (CPP-ACP) by incorporating different levels of propolis wax at 2%, 4%, and 6%. The study revealed a reduction in *S. mutans* biofilm mass in both saliva and gum eluent compared to the control groups of saliva and propolis (27). Meanwhile, 10% propolis was reported to have effective antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (28). Devitaningtyas et al also reported an inhibitory effect on the bacteria *Porphyromonas gingivalis*. by combining hydroxyapatite carbonate with 10% propolis (29). In addition, 10% propolis is known to help in the healing process of gingivitis in a rat model as reported by Suryono et al. Compounds in propolis such as CAPE, quercetin, and flavonoids, are thought to be able to affect lipoxygenase and cyclooxygenase. Inhibition of the lipoxygenase pathway by quercetin and flavonoids and cyclooxygenase by CAPE, can suppress prostaglandins at high concentrations, reduce the occurrence of vasodilation of blood vessels and blood flow will be reduced which causes the migration of leukocytes (PMN) to the area of inflammation is also reduced (30). Addition of 10% concentration of propolis to carbonate hydroxyapatite is reported to stimulate the growth of fibroblast cells and has the potential to regenerate alveolar bone (31). An in vivo study with 10% propolis showed stimulation of the gingivitis healing process in animals through reducing polymorphonuclear cells (PMN), increasing the number of fibroblasts, and stimulating the formation of angiogenesis (29).

Gelatin can play a crucial role in osteoblast proliferation by providing an extracellular matrix that supports the growth and differentiation of these cells. Additionally, gelatin can interact with growth factors that influence the osteoblast proliferation pathway. This may include its impact on cytokine activity, such as increasing the production of growth factors that stimulate osteoblast proliferation. Gelatin itself does not have the ability to directly inhibit the cytotoxic effects of propolis. However, in some studies, gelatin has been used as a carrier or encapsulation material for propolis, which can help reduce the cytotoxicity of propolis by forming a protective layer around cells or reducing propolis penetration into cells. In this context, gelatin may help reduce cell damage and allow cells to continue proliferating under the influence of propolis.

Regarding the components in propolis, two important compounds are Caffeic acid phenethyl ester (CAPE) and flavonoids. These compounds contribute to bone

regeneration; first, an effective component found in propolis is CAPE. CAPE stimulates bone regeneration by inhibiting osteoclast activity and increasing osteoblast activity. It has been studied *in vitro* and shows promise for bone healing (30). Second is flavonoids. These are major components in propolis. Some flavonoids in propolis exhibit antitumor and antiproliferative effects on cancer cells. Flavonoids can influence cellular signaling pathways involved in growth and cell proliferation (31). Cytotoxicity testing of materials on cell viability is carried out using the MTT test which can measure cell metabolic activity (18). Based on ISO 10993-5, a test material is declared to have cytotoxic potential if the cell viability percentage is less than 70%. A test material is said to be toxic if it has an inhibition value of more than 50 or what is known as IC50. IC50 is the concentration of test material that can cause the death of up to 50% of cells. The results of this research are expected to provide new information in the development of bone tissue engineering with hydroxyapatite-gelatine-propolis biomaterials.

The 10% propolis, 6% hydroxyapatite-propolis, and 6% hydroxyapatite-gelatine-propolis groups showed good cell viability with a percentage of more than 70% on day 1 (Figure 1). The biocompatibility of the hydroxyapatite group in this study falls in line with research done by Siswanto et al who also conducted MTT cytotoxicity tests on hydroxyapatite (32). Kattimani et al reported that hydroxyapatite only contains calcium and phosphate ions, so it does not cause unexpected local or systemic toxicity (33). According to Dubok et al, bioactive biomaterials allow organisms to form direct biochemical bonds with the surface of the material and free growth can occur (34). Its non-toxic properties seen in the 6% propolis group, this study falls in line with the literature which reports that clinical studies of propolis and its components on mice and humans are largely well tolerated and non-toxic unless given in large quantities (35). The viability of cells exposed to propolis depends on the concentration of propolis, where based on Fauzi et al, the lower the propolis concentration, the higher the cell viability (36).

The cytotoxic potential after 1 day of exposure in the exposure group containing 10% propolis was in accordance with Özan's research which reported mild cytotoxic properties of mouthwash containing 10% propolis (28). In addition, the cytotoxic potential of exposure to 10% hydroxyapatite-propolis and 10% hydroxyapatite-gelatine-propolis falls in line with Scatolini et al that reported the cytotoxic effect against CHO-k1 cell line from combining hydroxyapatite with Brazilian red propolis. The cytotoxic effect shown by propolis is thought to be related to the chemical composition of propolis which contains flavonoids, where the higher the number of flavonoids released by the ingredient, the higher the cytotoxic activity. Furthermore, it was found that the propolis extracts reduced the agglomeration

of hydroxyapatite, suggesting a negative effect on the physicochemical properties of the biomaterials (37). On the other hand, Grenho et al. (2015) conducted a study on the impact of Brazilian red and green propolis extracts on nanohydroxyapatite (nano-HA) biomaterials. Their findings demonstrated that nanoHA impregnated with propolis exhibited no cytotoxic effects fibroblasts cultures. Conversely, nanoHA impregnated with red propolis resulted in a rise in cellular metabolic activity when compared to untreated nanoHA.(38) Wijayanti et al. (2020) further illustrated that Brazilian propolis extract incorporated with carbonated hydroxyapatite induced the growth of NIH 3T3 fibroblast cells. Among the various concentrations examined, the highest concentration tested (10% wt propolis extract) exhibited the most significant growth, reaching approximately 125%, higher than the control group.(39) The activity profile of propolis can be influenced by various factors, such as the type of cells under study and the source of the propolis sample. The chemical composition of propolis may vary depending on the geographical region and climate characteristics, thereby affecting its specificity. (40, 41). In addition to the toxicity of the test material, other factors causing decreased viability on day 8 may be related to cell growth that has outgrown the experimental environment (96 well plate) (42).

Nurchayo, D. S. (2018) explained that CAPE plays a part in hindering osteoclastogenesis, reducing osteoblast apoptosis, increasing the amount of osteoblast, and the expression of fibroblast growth factor-2 (FGF-2), which is important for bone tissue regeneration. As with CAPE, Flavonoid also help in increasing the amount of osteoblast through biological effects, such as through its antioxidant and antimicrobial properties.

There is a factor which effects cell count and non-linear calcium deposition. That factor being ARS coloring and the use of ImageJ which can show fluctuating results. ARS coloring can be influenced by the loss of calcium deposition which is dissolved or washed away during the washing process, to remove excess ARS staining. The cell viability of all groups on day 8 was found to be less than 50%. Apart from the toxicity of the test material, this decrease in viability could be attributed to the excessive cell growth that surpassed the capacity of the experimental setting, specifically the 96-well plate. (40).

In research conducted by Sivasakthi et al, ARS assesses was used to assess calcium deposition and proliferation of osteoblast cells using intervals of days 1, 3, and 5. The results of the study showed an increase in calcium deposition and proliferation on days 1, 3, and 5(41). In the study conducted by Ying Pang et al used intervals of days 1, 3, and 7(42). In this study, time intervals of weeks were used, on days 7, 14, and 21.

This study is limited by its small sample size. Increasing

the sample size could be beneficial for future research to minimize measurement bias.

CONCLUSION

In conclusion, the results indicate that hydroxyapatite-gelatine-propolis at a concentration of 6% showed satisfactory biocompatibility, whereas hydroxyapatite-gelatine-propolis 10% exhibited toxic effects. The toxicity of hydroxyapatite-gelatine-propolis seems to be influenced by both the concentration and the duration of incubation. On the other hand, the elution of Hydroxyapatite-Gelatin-Propolis showed the potential to promote the proliferation and mineralization of osteoblast cells.

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