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Original Article

COMBINATION OF 10% PROPOLIS AND 0.8% HYALURONIC ACID GEL ACCELERATES OSTEOBLAST PROLIFERATION *IN VITRO*

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ABSTRACT

Objective: In treating periodontitis, bone regeneration can be promoted by signaling molecules and antibacterial properties. Hyaluronic acid induces signaling molecules; however, it has limited antibacterial properties. Propolis has the advantage of activating signaling molecules and possesses potent antibacterial properties. This investigation aimed to evaluate the impact of a combination of 10% propolis and 0.8% hyaluronic acid on the proliferation of MC3T3-E1 osteoblasts.

Methods: *In vitro* experiments were conducted in three groups, each with five technical replicates: group A received 10% propolis and 0.8% hyaluronic acid gel (n = 5), group B received 0.8% hyaluronic acid gel (n = 5), and group C received 2% Sodium Carboxy-Methyl Cellulose (CMC-Na) gel (n = 5). The osteoblast count was assessed on days 1, 3, and 7 using the Cell Counting Kit (CCK)-8 assay. Data were analyzed using Two-Way ANOVA and Tukey's High Significant Difference (HSD) test ($p<0.05$).

Results: The results indicated that the number of osteoblasts in group A was significantly higher than in groups B and C on days 3 and 7 ($p<0.05$). On day 3, the number of osteoblasts in group A was equivalent to that of group B on day 7 ($p>0.05$).

Conclusion: The combination of 10% propolis and 0.8% hyaluronic acid gel enhanced and expedited osteoblast proliferation in alveolar bone. The limitations of this study include the lack of *in vivo* validation and a relatively short observation period; hence, further studies are required prior to clinical translation.

Keywords: Osteoblast proliferation, Propolis, Hyaluronic acid, Bone regeneration

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INTRODUCTION

Periodontitis, a prevalent healthcare issue, can result in tooth loss and disability. It significantly affects mastication, aesthetics, and quality of life [1]. Irreversible alveolar bone loss is induced by periodontitis through the activation of osteoclast genesis [2]. The therapy for bone defects in periodontitis could involve tissue regeneration, necessitating a scaffold, signaling molecules, stem cells, and vascularization [3, 4].

The potential impact of extracellular matrix components, such as hyaluronic acid, on periodontal tissue regeneration has been the subject of studies by researchers in response to advancements in wound healing [5]. During wound healing, hyaluronic acid is produced to support tissue repair and modulate the activities of epithelial cells and fibroblasts. Multiple studies have shown that by combining hyaluronic acid with other materials can improve healing by reducing wound size, downregulating inflammation, and increasing the release of growth factors [6]. Nevertheless, hyaluronic acid has restricted antibacterial properties because it is a passive barrier preventing adherence and accumulation of biofilm. Therefore, hyaluronic acid could have better potential to enhance antibacterial efficacy when used in conjunction with other antimicrobials [7].

Propolis is a resinous substance created from plant components gathered by bees and processed with their enzymes and saliva, used for constructing their hives [8]. It is a natural material renowned for its antibacterial attributes. Additionally, it possesses anti-inflammatory, antioxidant, and tissue-regeneration properties [9]. Caffeic Acid Phenethyl Ester (CAPE) and flavonoids are the main constituents of propolis that have shown efficacy in antibacterial and bone regeneration [10, 11].

As a multifactorial chronic disease, treatment of periodontitis necessitates a comprehensive approach. The primary objective of periodontal therapy is to regenerate periodontal tissues following the elimination of infection and control of inflammation [12]. Integrating multifunctional therapies to eliminate infection and inflammation, while also stimulating the activity of precursor cells, would provide an environment favorable for tissue growth, differentiation, and morphogenesis. Therefore, several versatile composites have been developed for periodontitis [13, 14]. For instance, Wang *et al.* (2023) demonstrated that a hydrogel incorporating both antibacterial and osteogenic agents produced a synergistic effect, resulting in superior bone regeneration outcomes compared to each agent used alone in a periodontitis model [15]. This supports the rationale for our combination of propolis and hyaluronic acid gel, aiming to harness both antimicrobial and osteoinductive benefits.

The formulation developed in this study is in the form of gel, since it exhibits advantageous characteristics owing to their favorable physicochemical properties and capacity for localized drug delivery, hence enhancing efficacy and patient comfort [16]. Gel materials are relatively more convenient to apply to irregular bone defects, creating an extracellular matrix-like environment. This milieu promotes cell migration and proliferation [17–19].

Previous studies have demonstrated the therapeutic potential of combining propolis and hyaluronic acid in tissue regeneration. Khachatryan *et al.* (2023) developed hyaluronic acid-based films encapsulating propolis, showing strong antimicrobial activity and biocompatibility [20]. Similarly, Elsamman *et al.* (2024) reported that a propolis-hyaluronic acid nano-emulsion accelerated wound healing and collagen formation while reducing inflammation and bacterial load *in vivo* [21]. Ionescu *et al.* (2021) highlighted the synergistic benefits of incorporating propolis into hyaluronic acid-based electrospun scaffolds, enhancing cell proliferation and antimicrobial defense [22]. However, these studies employed varying concentrations and delivery systems, making it difficult to directly compare their outcomes or determine the optimal formulation. Therefore, we selected a standardized combination of 10% propolis and 0.8% hyaluronic acid, based on previous findings supporting their individual efficacy.

Several studies have highlighted the individual benefits of 10% propolis and 0.8% hyaluronic acid in promoting bone regeneration. The incorporation of 10% propolis into calcium hydroxyapatite has been shown to promote osteogenic markers, such as Transforming Growth Factor (TGF)-β [23] and osteocalcin [24], while simultaneously suppress Receptor Activatior of Nuclear Factor-κβ Ligand (RANKL) expression [25], and exhibit strong antimicrobial activity against the key periodontitis pathogens, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans in vitro* [26]. Meanwhile, 0.8% hyaluronic acid gel was administered as an adjunctive treatment for Open Flap Debridement (OFD) to treat intrabony defects in stage II/III, grade A/B periodontitis, with or without furcation involvement. The treatment exhibited a more significant increase in bone regeneration than OFD and placebo CMC-Na gel, as evaluated with Cone Beam Computed Tomography (CBCT) at 12 mo post-surgery [27]. Collectively, these findings provide a strong rationale for combining 10% propolis and 0.8% hyaluronic acid in our gel formulation. However, studies specifically investigating the synergistic interaction of these two agents at the stated concentrations in gel form have not yet been conducted. Therefore, this study was undertaken to explore their potential combined effect on bone regeneration, in line with our goal of developing a multifunctional agent with both antimicrobial and osteoinductive properties.

The evaluation of osteogenic effects and bone regeneration is contingent upon the proliferation of osteoblasts. Proliferation subsequently facilitates the maturation and mineralization of the bone matrix [28, 29]. Hence, it is essential to conduct a study to assess the influence of a composite gel containing 10% propolis and 0.8% hyaluronic acid on the proliferation of osteoblasts, to ascertain its capacity to stimulate bone formation.

MATERIALS AND METHODS

FKG and RSGM UGM Prof. Soedomo Research Ethics Commission granted Ethical Clearance No. 183/UN1/KEP/FKG-RSGM/EC/2023 for this study. This *in vitro* study is an experimental laboratory research. 10% propolis and 0.8% hyaluronic acid gel was obtained from Commanditaire Vennootschap (CV) Indoraya Internasional, with a measured viscosity of 18,326 cP. 0.8% hyaluronic acid gel was obtained as 1 ml Gengigel® Syringes. 2% Sodium Carboxy-Methyl Cellulose (CMC-Na) gel was prepared by mixing 2 mg of CMC-Na powder with 100 ml distilled water.

MC3T3-E1 cells (ECACC 99072810), at passage number 20, were cultured in 2,5x10^3 cells/well. The medium contains 89% α-Modified Eagle's Medium, 10% Foetal Bovine Serum, and 1% antibiotics-antimycotics. Incubation lasted 24 h at 37 °C, with 95% humidity and 5% CO_2. The cells were plated across three separate plates corresponding to different time points, with five technical replicates for each treatment group on every plate. The cells were then treated with test materials according to the following designation: group a received 10% propolis and 0.8% hyaluronic acid gel, group B received 0.8% hyaluronic acid gel, and group C received 2% CMC-Na gel. The gels were applied at a volume of 10 µl per well. The cells were cultured for 1, 3, and 7 d prior to observation.

The assessment of osteoblast proliferation was performed using the Cell Counting Kit (CCK)-8 assay. On d 1, 3, and 7, cells were infused with 10 µL of CCK-8 solution and re-incubated. Previous studies have reported acceptable CCK-8 assay incubation durations ranging from 1 h [30], 2 h [31], and 3 h [32]. After incubating for 3 h, the absorbance value was measured using a spectrophotometer at 450 nm wavelength. The absorbance value was converted into cell count using the normal curve equation.

The statistical analysis was performed using SPSS 25. Shapiro-Wilk normality test and Levene's homogeneity test preceded the Two-Way ANOVA hypothesis test. Finally, a Tukey High Significant Difference (HSD) Post Hoc test assessed intergroup differences. A p-value<0.05 was deemed statistically significant.

RESULTS

The proliferation of osteoblasts on day 1 did not exhibit any significant difference (p>0.05) across the groups, as seen in fig. 1. The proliferation of osteoblasts in group A was significantly higher (p<0.05) than that of groups B and C on d 3 and 7. There was no significant difference (p>0.05) in the proliferation of osteoblasts between group A on day 3 and group B on day 7.

Table 1: Mean and standard deviation of calculated cell counts according to test groups on d 1, 3, and 7

Observation time	n	Cell counts (x10^3 cells)			p-value
		10% propolis-0.8% hyaluronic acid gel (A)	0.8 hyaluronic acid gel (B)	2% CMC-Na gel (C)	
Day 1	5	4.304±0.232	4.076±0.516	3.645±0.260	<0.001*
Day 3	5	16.853±0.703	13.379±0.419	6.658±1.405	<0.001*
Day 7	5	20.198±0.320	15.879±1.454	11.538±1.130	<0.001*

n = number of technical replicates; CMC-Na = Sodium Carboxy-Methyl cellulose *p<0.05 is statistically significant

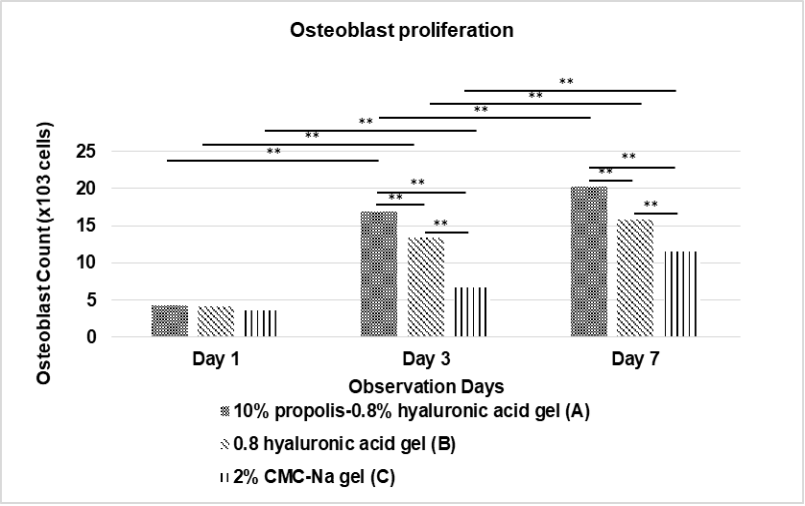

Fig. 1: Mean of osteoblast count in test groups, ** p<0.001

Table 2: Two-way ANOVA statistical summary

Source	η^2	p-value
Test Groups	0.925	<0.001*
Observation Day	0.977	<0.001*
Test Groups x Observation Day	0.839	<0.001*

η^2 = effect sizes; $\eta^2 \approx 0.01$ small effect; $\eta^2 \approx 0.06$ medium effect; $\eta^2 \geq 0.14$ large effect; $p<0.05$ statistically significant

DISCUSSION

Osteoblasts are cuboidal cells located on the surface of bones. They are essential for bone formation and bone defect regeneration [33, 34]. The MC3T3-E1 cell line is derived from osteoblast progenitors found in the calvarial bone of juvenile rodents [35]. Both calvarial and alveolar bones undergo intramembranous ossification, suggesting they share a similar osteoblast development pattern [36, 37]. Furthermore, the senescence pattern of MC3T3-E1 is identical to that of human cells. Therefore, this cell line is a suitable candidate to serve as a model for the study of alveolar bone regeneration [35]. The process of osteoblast development consists of three stages: cellular proliferation, deposition and maturation of the extracellular matrix, and ultimately the mineralization of the bone matrix [28]. The proliferation of osteoblasts is a crucial factor in bone regeneration. This stage could be stimulated by the introduction of active chemicals [38, 39]. The bioactivity of a substance could be measured by evaluating its proliferation over time [40].

Osteoblast proliferation stimulated by the test materials was observed at three time points: d 1, 3, and 7. On day 1, there was no significant difference in the number of osteoblasts across all groups, indicating that the three test materials had not affected the proliferation of osteoblasts, which served as the baseline. The negative control group C exhibited a substantial rise on d 3 and 7, indicating a typical cellular proliferation activity in the absence of any active agents. In groups A and B, a similar phenomenon of osteoblast proliferation was noticed. Specifically, proliferation was more significant on day 3 than on day 1 and even more remarkable on day 7. These findings align with the research done by Li *et al.* (2020), which showed that MC3T3-E1 cells exhibited accelerated proliferation starting on day 3 [41]. Overall, all test groups had a physiological pattern of proliferation that was consistent with the theory by Beck *et al.* (2001). Based on their premise, the proliferation of murine osteoblast cell line MC3T3-E1 began on day 3 and peaked on day 7 [42].

Osteoblastogenesis is regulated by a sequence of signaling pathways that, in turn, stimulate transcription factors and trigger the production of osteogenic markers. Signaling molecules involved in the proliferation of osteoblasts include Bone Morphogenetic Protein (BMP)-2, TGF-β, and Fibroblast Growth Factor (FGF)-2. The expression of these molecules could be increased by propolis and hyaluronic acid [39, 43]. Propolis enhances the expression of BMP-2 [40], TGF-β, and FGF-2 [44]. Hyaluronic acid could also boost osteoblast proliferation by upregulating the release of TGF-β [45]. Furthermore, osteoblast CD44 receptors could form HA-CD44 complexes with hyaluronic acid. This combination would increase the transcription factors required to initiate cell mitosis, accelerating cell proliferation [46].

The proliferation of osteoblasts in group A was 2.5 times greater on day 3 than in group C, and 1.5 times greater on day 7. Group B also exhibited a significant increase in osteoblast proliferation than group C. The proliferation of osteoblasts could be significantly increased by using 10% propolis and 0.8% hyaluronic acid as signaling molecules stimulants, in contrast to the negative control 2% CMC-Na, which contained no active substances [39].

The superior osteoblast proliferation observed in our study with the combination of propolis and hyaluronic acid aligns with previous findings demonstrating the individual osteogenic effects of those substances. For instance, Somsanith *et al.* (2018) demonstrated that propolis enhanced MC3T3-E1 cell proliferation and alkaline phosphatase activity *in vitro*, and improved osseointegration *in vivo* by upregulating osteogenic markers, such as BMP-2 and BMP-7, while simultaneously reducing pro-inflammatory cytokines, like IL-1β and TNF-α [40]. Similarly, Asparuhova *et al.* (2020) reported that hyaluronic acid stimulated osteoprogenitor cell proliferation and early osteogenic signaling through the TGF-β and Erk pathways, reinforcing its role in modulating early bone healing responses [45]. While our data show that the combination treatment yielded greater proliferation than HA alone, it is important to carefully note that differences in methodology may affect comparability.

On d 3 and 7, group A exhibited 1.25 times the proliferation of osteoblasts as group B. In addition, osteoblast proliferation in group A on day 3 was not significantly different from that in group B on day 7. This study determined that osteoblast proliferation is enhanced by the combination of propolis and hyaluronic acid, as opposed to either substance alone. This effect may be attributed to the increased release of signalling molecules by propolis' active components, which include flavonoids and CAPE. The heightened release of signaling molecules ultimately accelerates osteoblast proliferation [47].

The increased osteoblast proliferation resulting from applying the 10% propolis and 0.8% hyaluronic acid gel may be attributed to molecular interactions between the two substances. Propolis flavonoids could bind with glucuronic acid derived from hyaluronic acid through esterification [48]. Esterification optimizes the solubility of flavonoids in lipophilic environments, enabling them to penetrate cell membrane phospholipids and increase intracellular bioavailability [49]. Flavonoids within cells interact with the Smad 1/5/8 molecules to promote the synthesis of transcription factors essential for osteoblast proliferation [50]. However, the esterification mechanism in our formulation remains speculative, and further confirmation with Fourier-Transformed Infrared Spectroscopy (FTIR) or Nuclear Magnetic Resonance (NMR) is warranted.

This study compared the osteogenic effects of 10% propolis combined with 0.8% hyaluronic acid gel to only 0.8% hyaluronic acid. While the osteogenic effects of 10% propolis have been widely examined, it is also essential to further explore the osteogenic effect and biocompatibility of the combined 10% propolis and 0.8% hyaluronic acid gel. Future studies should compare the effectiveness of the two gels with that of using only either substance alone. These investigations aim to examine propolis and hyaluronic acid's synergistic effect and biocompatibility.

The efficacy of regenerative treatments relies on four crucial elements: scaffold, signaling molecules, stem cells, and vascularisation [3]. Active compounds in 10% propolis and 0.8% hyaluronic acid release signaling molecules [39, 45]. A gel of the two active compounds creates an extracellular matrix-like environment in tissues and subsequently aids cell migration and proliferation [18]. Nevertheless, gel-based scaffolds are often pliable and lack the mechanical strength needed for bone regeneration. Conversely, stability and space maintenance are crucial for bone regeneration.

Moreover, the challenge in treatments involving signaling molecules lies in the requirement to gradually release active chemicals to achieve optimal results in tissue regeneration. This may be accomplished by regulating the retention and release profile of the active compounds while also ensuring the preservation of the structure and stability of the scaffold. Thus, the degradation of 10% propolis and 0.8% hyaluronic acid gel must be studied to establish its utility as a scaffold for bone regeneration. This includes having adequate mechanical strength to maintain spatial integrity and a degradation profile that allows the gradual release of active substances [13, 51].

This study has several limitations. First, the absence of FTIR or High-Performance Liquid Chromatography (HPLC) analyses prevented confirmation of the chemical stability and potential interactions between propolis and hyaluronic acid. Additionally, future studies should include Western blot assays and downstream osteogenic markers such as alkaline phosphatase activity or osteocalcin expression to better elucidate the mechanism behind the observed proliferation. The observation period was limited to seven days due to the spatial constraints of the 96-well plates; however, bone regeneration is a complex and prolonged process. Therefore, *in vivo* studies are needed to evaluate the long-term regenerative effects of this gel formulation.

CONCLUSION

In conclusion, this study demonstrated that the combination of 10% propolis and 0.8% hyaluronic acid gel significantly enhanced osteoblast proliferation compared to hyaluronic acid alone. While this *in vitro* finding is promising, it does not directly translate to *in vivo* bone regeneration. Further studies are needed to assess the gel's degradation profile, biocompatibility, and long-term regenerative potential. In additional, mechanistic analyses such as Western blotting and downstream osteogenic assays, along with extended *in vivo* evaluations, are essential to fully understand and validate the therapeutic potential of this formulation.

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AUTHORS CONTRIBUTIONS

JW: Performed the Experiments, Writing, Analysis Interpretation; RS: Writing, Critically Revised the Manuscript; S: Research Conception

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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