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Content of this issue

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ORIGINAL ARTICLE

Antibacterial and cytotoxic effects of fresh bovine amniotic membrane with hydroxyapatite (BAM-HA): a laboratory experiment

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ABSTRACT

Introduction: Bacterial infections, particularly by *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* and Porphyromonas *gingivalis* (*P. gingivalis)*, can worsen alveolar bone resorption after tooth extraction. The capability of Bovine Amniotic Membrane-Hydroxyapatite (BAM-HA) biocomposite to reduce this resorption has been explored. However, before clinical use, cytotoxicity testing is imperative to ensure its biocompatibility. The aim of the study was to analyzed both the antibacterial effects and cytotoxicity of the BAM-HA biocomposite to ensure its suitability for clinical use biocompatibility of the BAM-HA biocomposite before its clinical application. **Methods:** The laboratory-based research involved testing BAM combined with HA powder in 4:1 and 4:2 ratios via freeze-drying and underwent antibacterial tests against *A. actinomycetemcomitans* and *P. gingivalis*, using the plate count method. Cytotoxicity tests were performed on HGF cells, including negative control, positive control, BAM-HA (4:1), and BAM-HA (4:2) groups, with statistical analysis conducted using One-Way ANOVA and Post Hoc Bonferroni and Tukey tests. **Results:** Antibacterial tests against *A. actinomycetemcomitans* revealed significant reduction in colony count with BAM-HA ratios 4:1 (129.0 ± 12.7 CFU/mL) and 4:2 (77.3 ± 15.5 CFU/mL) compared to the negative control (186.6 ± 27.5 CFU/mL). Similar reductions were observed for *P. gingivalis*, with BAM-HA ratios 4:1 (51.3 ± 6.6 CFU/mL) and 4:2 (3.1 ± 1.5 CFU/mL) compared to the negative control (117.3 ± 22.0 CFU/mL). Cytotoxicity tests showed no significant differences in HGF cell viability and IC50 values between the negative control and BAM-HA (4:1) or BAM-HA (4:2) groups. **Conclusion:** The BAM - HA biocomposite shows antibacterial effects against *A. actinomycetemcomitans* and *P. gingivalis*. Moreover, BAM - HA ratios of 4:1 and 4:2 do not induce cytotoxic effects on human gingival fibroblasts, suggesting potential biocompatibility for clinical applications.

KEYWORDS

A. actinomycetemcomitans, antibacterial effects, BAM-HA biocomposite, cytotoxicity, *P. gingivalis*

INTRODUCTION

Tooth extraction leads to alveolar bone resorption within the first six months post-extraction causing structural changes in vertical and horizontal dimensions.[1] Alveolar bone loss during the initial three months post-extraction reaches 3.87 mm horizontally and 1.67 mm vertically.[2] Alveolar bone resorption poses challenges during the application of dental restorations and implant placement, impacting aesthetics.[3,4] Therefore, it is necessary to take action to prevent the

trauma caused by bone resorption during extraction through socket preservation. Socket preservation is a procedure intended to maintain bone volume after tooth extraction.[3] Socket preservation may involve application of materials like Bovine Amniotic Membrane (BAM). BAM can be found in bovine placentas, and shares a chemical composition resembling the human bone.[5,6] With its potential for tissue repair and regeneration, BAM accelerates re-epithelialization and wound healing. BAM exhibits osteoinductive properties and antibacterial effects due to antimicrobial peptides such as defensin, elafin, and SLPI (Secretory Leukocyte Protease Inhibitor).[7,8] Furthermore, BAM has been proven to accelerate epithelialization, possesses strong anti-inflammatory, anti-angiogenic, and analgesic effects. The application of BAM is expected to reduce alveolar bone resorption.[9]

Combining amniotic membrane and osteoconductive bone-forming material will increase bone regeneration. BAM can be combined with hydroxyapatite (HA) in the form of $Ca_{10}(PO_4)_6(OH)_2$.[10] The biocomposite of BAM-HA has shown promise in preserving bone volume.[5] HA is considered as the most stable calcium phosphate salt that contains carbonates (CO_2^{-3}), sodium (Na^+), magnesium (Mg^{2+}), iron (Fe^{2+}), fluoride (F^-), silicates, and chlorides (Cl^-). HA is extensively utilized as a biomaterial in bone tissue replacement and repair due to its excellent osteoconductive properties, lack of toxicity, and favorable biocompatibility.[11] An in vivo study found that the biocomposite combination of HA and collagen, when implanted into the bone substance, is absorbed by osteoclasts through phagocytosis.[12]

In this study, BAM and HA were combined in ratios of 4:1 and 4:2, forming a novel biocomposite sponge. This material was made in ratios 4:1 and 4:2 to enhance the properties of the combination material from previous research and also to align with existing commercial products, thereby improving its ability to prevent alveolar bone resorption. This combination aimed to maximize the individual functions of BAM and HA.[13] Unlike commercial materials using porcine collagen, BAM-HA biocomposite serves as a halal alternative for socket preservation, supporting alveolar bone regeneration.[14] BAM-HA biocomposite is not only beneficial for bone regeneration, but also plays a crucial role in healing post-extraction wounds, which involve alveolar bone, periodontal ligaments, and gingiva.[15] One inhibiting factor in this healing process is infection, leading to bacterial colonization and increased inflammation in periodontal ligaments and cementum.[15,16]

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are types of bacteria present in the oral cavity that influence the post-tooth extraction healing process. *A. actinomycetemcomitans* and *P. gingivalis*, both are found in subgingival pockets.[17] A*. actinomycetemcomitans* is a gram-negative, facultatively anaerobic, and non-motile bacterium.[18] *P. gingivalis* is a gram-negative, non-motile, and obligate anaerobic bacterium.[19] Their overabundance increases osteoclastic activity, decreases osteoblastic activity, and slows healing processes.[20] Therefore, it is necessary to conduct antibacterial tests to prevent those bacteria to worsen the alveolar bone resorption process.

In addition to its antibacterial properties, BAM-HA must exhibit non-toxicity towards human gingival fibroblasts (HGF), which is essential for wound healing.[21] Since the toxic exposure to fibroblasts can induce apoptosis or necrosis, HGF play a crucial role in responding to oral pathogens, experiencing excessive apoptosis during inflammatory conditions, and possessing the ability to initiate inflammatory processes. As a result, they contribute to the healing of gingival tissue damage during post-tooth extraction recovery processes. HGF, crucial for responding to oral pathogens, might experience increased apoptosis during inflammation, leading to tissue damage.[22]

The combination of BAM-HA is expected to become a good composition for possessing antibacterial properties, potentially accelerating bone resorption. Additionally, due to its chemical composition and inherent qualities, BAM-HA is

expected to be antibacterial and free from cytotoxic effects on HGF. As a result, any BAM-HA material designed for oral applications should ensure safety across various oral tissues, including mucosa, gingiva, pulp, and bone. Moreover, it is essential to do cytotoxicity testing to assess potential risks and guarantee biocompatibility. Based on the description provided, the aim of this study was to evaluate the antibacterial effects of BAM-HA biocomposite (ratios 4:1 and 4:2) on *A. actinomycetemcomitans* and *P. gingivalis* while analyzing the cytotoxic effects on HGF.

In contrast to earlier research, this study prioritized the safety assessment of BAM-HA across various oral tissues, integrating thorough antibacterial effects and cytotoxicity testing to ensure biocompatibility. The study also emphasized the necessity for materials used in the oral cavity to be safe for all oral tissues, including mucosa, gingiva, pulp, and bone, without containing soluble toxic substances that could enter the bloodstream and induce systemic toxic responses. The aim of the study was to analyzed both the antibacterial effects and cytotoxicity of the BAM-HA biocomposite to ensure its suitability for clinical use and biocompatibility of the BAM-HA biocomposite before its clinical application.

METHODS

This study was in vitro experimental laboratory design utilizing the post-test-only control group design in vitro. The process began by cleaning fresh BAM from blood clots and washing it four times for 10 minutes using a 0.05% saline solution. The BAM was further washed with aquadest until the saline solution was clear, then cut into pieces and mixed with NaCl in a 1:1 ratio. This mixture was then homogenized into amnion porridge. In order to create BAM-HA biocomposite at a 4:1 ratio, 20 mL of amnion porridge was mixed with 5 mL of HA powder. The 4:2 ratio combined 20 mL of amnion porridge with 10 mL of HA powder.

The resulting mixture was homogenized, placed in a 10 cm diameter container, and frozen at -80°C for 24 hours. Subsequently, freeze-drying was performed for 48 hours at 100°C. The BAM-HA combination was sterilized through a 25 kGy gamma irradiation and stored in conical tubes at a low temperature, around 2°C-8°C.

The study samples included BAM-HA in sponge form with ratios of 4:1 and 4:2 and Bio-Oss Collagen. Additionally, pure cultures of A. actinomycetemcomitans ATCC 29522 and P. gingivalis ATCC 33277 from MiCORE Laboratory stock were used. BAM-HA, at a quantity of 100 µg, was applied to human gingival fibroblasts (HGF) at a density of 1 x 104 cells/well. In the antibacterial testing, BAM-HA and Bio-Oss Collagen (Geistlich, UK) sponge samples were cut into circles with a 5 mm diameter and sterilized with 25 kGy gamma radiation. Bio-Oss collagen was used as a positive control due to its popularity as a socket preservation material. This material has the same structure with BAM-HA biocomposite and has been proven to have a good capability in bone regeneration.

The culture media for both bacteria were prepared using BHI-B medium by dissolving 3.7 grams of BHI-B powder in 100 mL of sterile distilled water in an Erlenmeyer flask covered with aluminum foil. The mixture was autoclaved at 121°C for 15 minutes to achieve homogeneity and sterility. Subsequently, *A. actinomycetemcomitans* ATCC 29522 and *P. gingivalis* ATCC 33277 stocks from the MiCORE Laboratory were drawn using sterile needles and introduced into the broth. Both bacteria were homogenized with a vortex, and the solution was incubated in an anaerobic jar (Oxoid) at 37°C for 24 hours. Additionally, BHI-A medium (Brain Heart Infusion Agar, Oxoid) was prepared by dissolving 9.25 grams of BHI-B powder (Oxoid) and 3.75 grams of bacteriological agar powder (Himedia) in 250 mL of sterile distilled water in an Erlenmeyer flask.[23]

After sterilization at 121°C for 15 minutes, the solution was poured into sterile petri dishes (4 mm thickness) and allowed to solidify.[24] Bacterial cultures were then prepared in 10 mL of sterile PBS solution with turbidity adjusted to

McFarland standard 0.5, corresponding to 1.5x10^8 CFU/mL. The obtained bacterial cultures were diluted tenfold, and 375 µL of bacterial culture was mixed with 1125 µL of BHI-B medium in a microplate for each group.

Bacterial suspensions were added to microplate wells for each sample group. After 24 hours of incubation at 37°C, 4 µL of each sample was taken, diluted 1,000,000 times with sterile PBS, and streaked onto agar plates. The plates were incubated r an additional 24 hours at 37°C. Antibacterial activity was assessed based on the total plate count and CFU/mL.[25] Data were processed using Statistical Product and Service Solution (SPSS), with normality tested using the Shapiro-Wilk test. If normality was confirmed, one-way ANOVA was conducted, followed by post hoc testing using Bonferroni if $p<0.05$.

For cytotoxicity testing, BAM-HA biocomposite was extracted from its storage media (conical tube). Subsequently, the material was cut using a surgical knife, and its weight was accurately measured using a digital scale. The desired weight for each well was set at 100 µg of the BAM-HA sample.

Human Gingival Fibroblast (HGF) cells underwent cell culture processes. HGF cells were propagated in dishes containing 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. The incubation took place at 37°C in a humidified environment with 5% CO_2. Every two days, the cell culture medium was replaced, and the cells reached optimal density after 7 days. Cells were rinsed with Hank's solution and incubated with trypsin for 4 minutes. A mixture of 5 mL DMEM and FBS was added beneath the cell monolayer to facilitate cell detachment. Homogenized HGF cells were then placed in a 96-well microplate at a density of 2 x 10^5 cells/mL, and incubated for 24 hours.[26]

HGF cells were harvested when reaching approximately 80% cell density. The harvest involved removing the HGF cell culture medium from the CO_2 incubator, followed by media disposal using a micropipette. Next, 3 mL of phosphate-buffered saline (PBS) was added to the cell culture, and the solution was incubated. The PBS solution was then aspirated with a micropipette and discarded. Subsequently, 3 mL of trypsin solution was introduced into the cell culture medium, followed by a 5-minute incubation in the CO_2 incubator. Afterward, cells were examined under a microscope to evaluate their condition. The subsequent steps included inactivation, resuspension, and observation under a microscope using PGS, trypan blue, and the suspended cell solution.[27,28]

The study comprised four groups: negative control group, positive control group, and two treatment groups. The negative control group consisted of untreated HGF, the positive control group involved 0.1 mg of Bio-Oss Collagen, and the treatment groups were treated with either BAM-HA composite in a 4:1 or 4:2 ratio. In the negative control group, 10,000 cells/well of HGF were placed in a 96-well microplate, while in the positive control group, 0.1 mg of Bio-Oss Collagen was applied to a 96-well microplate containing 10,000 cells/well of HGF. In the BAM-HA 4:1 group, treatment included 10,000 cells/well of HGF and 0.1 mg of BAM-HA composite in a 4:1 ratio in a 96-well microplate. Similarly, in the BAM-HA 4:2 group, treatment involved 10,000 cells/well of HGF and 0.1 mg of BAM-HA composite in a 4:2 ratio in a 96-well microplate. Each treatment group was replicated seven times, and the microplate was placed in an incubator at 37°C for 24 hours.

Subsequently, the cell growth medium was removed, and washing was performed using 100 µl of phosphate-buffered saline (PBS). Then, 100 µL of a solution containing 10 µL of CCK-8 with 90 µL of PBS was added to each well. The microplate was incubated for approximately 4 hours at 37°C and mechanically stirred using a plate shaker for 5 minutes to ensure complete dissolution of formazan crystals. Further washing with Dimethyl sulfoxide (DMSO) was carried out. Living HGF cells were stained with formazan, resulting in an orange color, while dead cells did not show orange coloration. Formazan absorbance was read using a 96-well Microplate reader at a wavelength of 450 nm to obtain Optical

Density (OD) values. The more intense the formazan color, the higher the absorbance value, indicating a higher number of viable cells.[29]

The obtained data were compared with ISO 10993-5 standards. According to ISO 10993-5, cell viability percentages above 80% are considered non-cytotoxic; 80% - 60% indicates low cytotoxicity; 60% - 40% suggests moderate cytotoxicity, and below 40% indicates high cytotoxicity.[30] A higher IC50 value corresponds to lower material toxicity. The acquired data were analyzed using the Kolmogorov-Smirnov normality test, Levene's test for variance homogeneity, and One-way ANOVA analysis with a significance level of $p<\alpha$ ($\alpha=0.05$). In case of differences, post-hoc Tukey tests were conducted.

RESULTS

The antibacterial analysis of *A. actinomycetemcomitans* revealed round white colonies on the agar medium. The petri dish was divided into three sections for repetitions, and bacterial colony counting was performed in each section. The negative control group exhibited more bacteria than other groups (Figure 1A). The BAM-HA 4:1 group showed more rounded shapes than the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups (Figure 1B). The BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups had similar quantities of rounded shapes (Figure 1C, 1D).

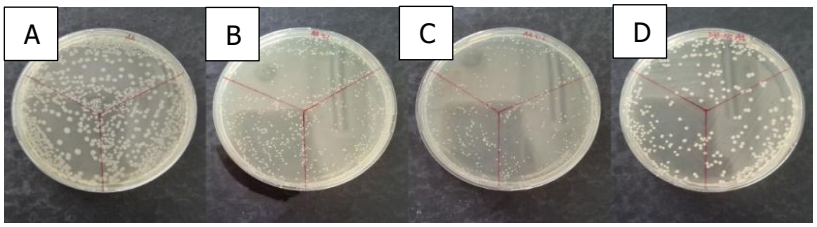
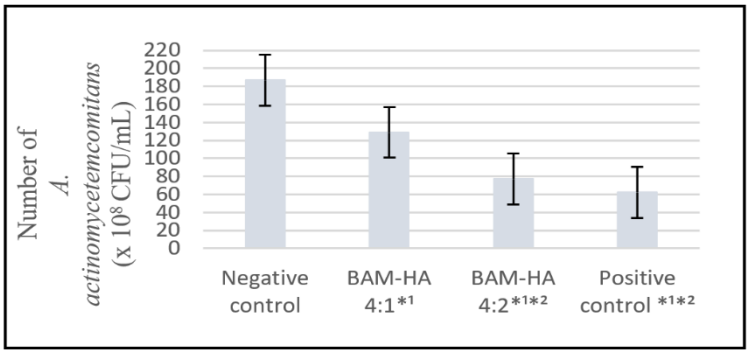


Figure 1. illustrates colonies of A. actinomycetemcomitans in Petri dishes (A) bacterial colonies without treatment (negative control), (B) bacterial colonies after treatment with BAM-HA biocomposite at a 4:1 ratio, (C) bacterial colonies after treatment with BAM-HA biocomposite at a 4:2 ratio, and (D) bacterial colonies after treatment with Bio-Oss Collagen (positive control).

The average bacterial colony counts in the four test groups were (186.6 ± 27.5)x10^8 CFU/mL, (129.0±12.7)x10^8 CFU/mL, (77.3±15.5)x10^8 CFU/mL, and (62.3±2.5)x10^8 CFU/mL, respectively (Graphics 1). The percentage reduction in colony count compared to the negative control for the three test groups was 30.8, 58.5, and 66.6%, respectively.



Graphics 1. Average colony of *A. actinomycetemcomitans* in antibacterial testing
*1 There were significant differences with negative control groups
*2 There were significant differences with BAM-HA 4:1 groups

Statistical analysis using one-way ANOVA and post hoc Bonferroni tests indicated significant differences between the negative control and BAM-HA 4:1, 4:2, and Bio-Oss Collagen (positive control) groups, with $p < 0.05$. The BAM-HA 4:1 group also showed a significant difference ($p < 0.05$) compared to the BAM-

HA 4:2 and Bio-Oss Collagen (positive control) groups. However, the BAM-HA 4:2 group did not exhibit significant differences from the Bio-Oss Collagen (positive control) group, with a p-value of 1 (Graphics 1 & Table 1).

Table 1. Statistical Analysis of one-way ANOVA with post-hoc Bonferroni Test for the Antibacterial Effect of BAM-HA Biocomposite on *A. actinomycetemcomitans*

Control Group	Negative Control	BAM-HA 4:1	BAM-HA 4:2	Positive Control
Negative		0.020*	0.001*	0.001*
BAM-HA 4:1			0.036*	0.008*
BAM-HA 4:2				1
Positive				

In the antibacterial test against *P. gingivalis*, observations revealed rounded white colonies on the agar medium. The negative control group exhibited more bacteria than other groups (Figure 2A). The BAM-HA 4:1 group showed significantly more rounded shapes than the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups (Figure 2B). The BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups had minimal and almost equal quantities of bacteria (Figure 2C, 2D).

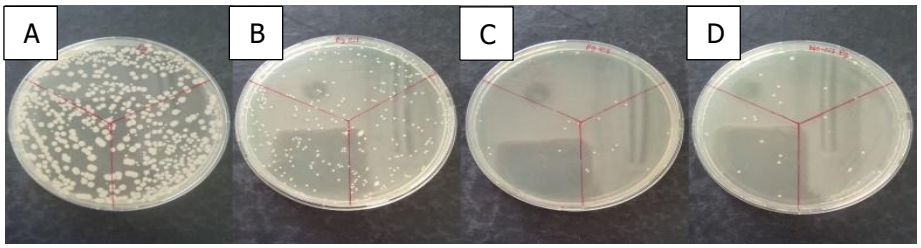
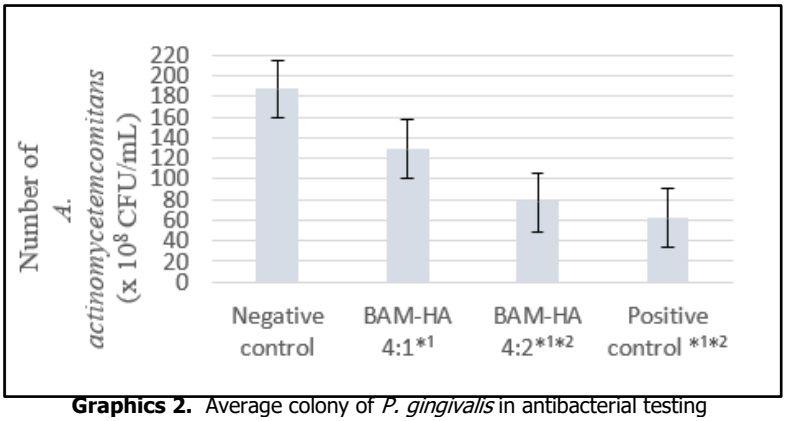


Figure 2. Colonies of *P. gingivalis* in Petri dishes (A) bacterial colonies without treatment (negative control) (B) bacterial colonies after treatment with BAM-HA biocomposite 4:1 (C) bacterial colonies after treatment with BAM-HA biocomposite 4:2 (D) bacterial colonies after treatment with Bio-Oss Collagen (positive control)

The average bacterial colony counts in the four test groups were (117.3±22.0) x 10^8 CFU/mL, (51.3± 6.6) x 10^8 CFU/mL, (3.1±1.5) x 10^8 CFU/mL, and (4.5±1.3)x10^8 CFU/mL, respectively (Table 4). The percentage reduction in colony count compared to the negative control for the three test groups was 56.2%, 97.3%, and 96.1%, respectively (Graphics 2).

Statistical analysis using one-way ANOVA and post hoc Bonferroni tests indicated significant differences ($p<0.05$) between the negative control with BAM-HA 4:1, 4:2, and Bio-Oss Collagen (positive control) groups. The BAM-HA 4:1 group also exhibited a significant difference ($p<0.05$) compared to the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups. However, the BAM-HA 4:2 group did not differ significantly from the Bio-Oss Collagen (positive control) group, with a p-value of 1 (Graphics 2 & Table 2).

For the Cytotoxicity Test, during the research, two visual observations of HGF were conducted under a microscope. The first observation was made before treatment with Bio-Oss Collagen, BAM-HA ratio 4:1, and BAM-HA ratio 4:2 on HGF. This observation indicated the success of cell culture and suitable conditions for proceeding to the following research phase.



Graphics 2. Average colony of *P. gingivalis* in antibacterial testing
*1 There were significant differences with negative control groups
*2 There were significant differences with BAM-HA 4:1 groups

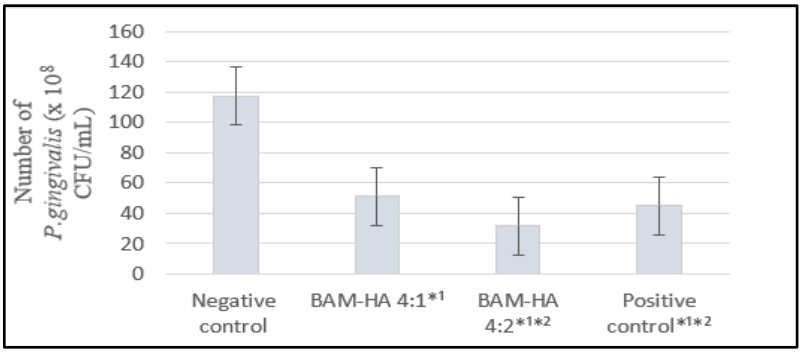
Table 2. Statistical Analysis of one-way ANOVA with post-hoc Bonferroni Test for the Antibacterial Effect of BAM-HA Biocomposite on P. gingivalis

Control Group	Negative Control	BAM-HA 4:1	BAM-HA 4:2	Positive Control
Negative		0.001*	0.001*	0.001*
BAM-HA 4:1			0.006*	0.007*
BAM-HA 4:2				1
Positive				

Subsequent observations were made after cells were treated with Bio-Oss Collagen, BAM-HA ratio 4:1, BAM-HA ratio 4:2, and incubated for 24 hours before CCK-8 administration. This observation reflected the initial step in evaluating cell responses to treatment before proceeding to the next step, CCK-8 testing and cell viability analysis to understand the toxic effects of these materials on HGF more deeply.

Visual observations of cell viability continued after applying the CCK-8 reagent and subsequent one-hour incubation. Further identification of the color change, reflecting the activity of live cell dehydrogenase enzymes, was more accurately performed using a microplate reader. The results obtained were optical density (OD) values.

The Optical Density (OD) values from the cytotoxicity test of BAM-HA ratio 4:1, BAM-HA ratio 4:2, and Bio-Oss Collagen on HGF were then converted using formulas for cell viability percentage and cell inhibition percentage. Cell viability and IC50 values from each test group were obtained (Graphics 3).



Graphics 3. Cytotoxicity Test Results of BAM-HA Biocomposite
* There were significant differences with negative control groups

Graphics 3 illustrates the average and standard deviation values of cell viability in various groups. The cell viability value for the negative control group was 100±3.35, while the positive control group had a value of 79.30±4.92. In the BAM-HA 4:1 and 4:2 treatment groups, cell viabilities reached 80.63±3.28 and 90.69±3.28. Graphics 3 also provides information on the IC50 values for each test

group: positive control group (78.20), BAM-HA 4:1 treatment group (81.91), and BAM-HA 4:2 treatment group (89.58).

Statistical analysis indicated normal data distribution ($p>0.05$) based on the Kolmogorov-Smirnov normality test for all sample groups. The homogeneity of variance test using Levene's test showed homogeneous variance data ($p>0.05$) with a p-value of 0.362. One-way ANOVA resulted in a significance of 0.0001 ($p<0.05$), indicating a significant difference in the average cell viability values among sample groups.

The post-hoc Bonferroni test showed a significant difference in cell viability values between the negative and positive control groups. There was no significant difference in cell viability values between the control group and BAM-HA 4:1 and 4:2 groups, indicating no significant difference in cell viability among these three groups. The same applied to cell viability values between the Bio-Oss Collagen group and the BAM-HA 4:1 and 4:2 groups.

DISCUSSION

Based on the antibacterial tests conducted on samples, there was a decrease in the quantity of *A. actinomycetemcomitans* and *P. gingivalis* bacteria in the BAM-HA 4:1 and 4:2 (Graphics 1 and Graphics 2). These findings were consistent with the previous research where bone scaffold materials with hydroxyapatite as the main component exhibited antibacterial effects against gram-negative bacteria.[31] Other research by Cunniffe et al also indicated that hydroxyapatite exhibits antibacterial properties.[32] Furthermore, the reduction percentage in the quantity of *P. gingivalis* bacteria in BAM-HA 4:1 and 4:2 biocomposites was more significant compared to *A. actinomycetemcomitans* bacteria. This difference is due to variations in the composition of cell wall structures and lipopolysaccharides (LSP) in each bacterium, causing different sensitivities to the biocomposite.[33]

This study also showed that applying Bio-Oss Collagen material can reduce the growth of both *A. actinomycetemcomitans* and *P. gingivalis* bacteria. Bio-Oss Collagen is widely used in socket preservation and consists of 90% deproteinized bovine bone mineral (DBBM) and 10% collagen. The main component of DBBM is hydroxyapatite, indicating similar antibacterial mechanisms to the BAM-HA biocomposite.[34] Therefore, the antibacterial test results suggest that the BAM-HA biocomposite has capabilities comparable to Bio-Oss Collagen in reducing bacterial growth.

Furthermore, regarding cytotoxicity, this study found that cell viability in the negative control group reached 100%. Correspondingly, findings from a study by Neto et al.[35] exhibited comparable results, indicating 100% viability in the negative control cells group. This viability value was obtained in the cytotoxicity test using the CCK-8 Assay method, reflecting the cell survival after treatment. Cells in this group were not exposed to foreign substances that could affect their metabolism and survival.[36] Consequently, mitochondrial dehydrogenase enzymes in the cells reduced the CCK-8 reagent (WST-8) to form orange-colored formazan. The formazan concentration values indicated perfect cell viability. The negative control group did not have an IC_{50} value because cells in this group were not exposed to any foreign substances that could cause a decrease in the number of living cells, making cell viability the reference baseline or normal condition that does not require IC_{50} calculation.

The viability values for the BAM-HA 4:1 and 4:2 treatment groups, as indicated in Graphics 3, were 80.6% and 90.6%, with concomitant elevated IC50 values standing at 81.91 and 89.58. This result is in concordance with a study by Octarina et al.,[37] wherein a significantly high fibroblast viability of 98.14% was observed for BAM-HA with a 35:65 ratio. Lower cytotoxicity levels can be associated with high IC_{50} values. It means that the biomaterial has minimal negative impact on the cells' survival ability. The cytotoxicity test results in the positive control group showed a cell viability value lower than the treatment

groups, at 79.3%. Consistent with previous research, it is known that the viability of Bio-Oss Collagen cells gradually decreases.[38] The gradual decrease in cell viability, indicating cell death due to exposure to foreign substances, can be triggered by various factors, one of which is related to the content of the biomaterial. Therefore, using biomaterial with a specific viability percentage should be limited in certain exposures to remain within the body's tolerance range. This research was confined to laboratory investigations. Further studies targeting osteoblast cells and animals are essential to validate BAM-HA biocomposite efficacy in preventing alveolar bone resorption.

CONCLUSION

The BAM-HA biocomposite has antibacterial effects on both *A.actinomycetemcomitans* and *P. gingivalis* bacteria. The biocomposite with ratios of 4:1 and 4:2 exhibits antibacterial effects against both bacteria. This study also shows that BAM-HA ratios of 4:1 and 4:2 do not have cytotoxic effects on human gingival fibroblasts. However, further supporting tests are required, including in vivo studies on animal models and clinical trials to assess the effects of the BAM-HA biocomposite. Additionally, further in vitro research involving osteoblasts is recommended, followed by experiments on animals and clinical trials to verify the biocompatibility and impacts of the biomaterial. Implications of this research: it is hoped that this material can prevent alveolar bone resorption after extraction.

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Antibacterial and cytotoxic effects of fresh bovine amniotic membrane with hydroxyapatite (BAM-HA): a laboratory experiment

by Octarina FKG

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ORIGINAL ARTICLE

Antibacterial and cytotoxic effects of fresh bovine amniotic membrane with hydroxyapatite (BAM-HA): a laboratory experiment

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ABSTRACT

Introduction: Bacterial infections, particularly by *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) and Porphyromonas *gingivalis* (*P. gingivalis*), can worsen alveolar bone resorption after tooth extraction. The capability of Bovine Amniotic Membrane-Hydroxyapatite (BAM-HA) biocomposite to reduce this resorption has been explored. However, before clinical use, cytotoxicity testing is imperative to ensure its biocompatibility. The aim of the study was to analyzed both the antibacterial effects and cytotoxicity of the BAM-HA biocomposite to ensure its suitability for clinical use biocompatibility of the BAM-HA biocomposite before its clinical application. **Methods:** The laboratory-based research involved testing BAM combined with HA powder in 4:1 and 4:2 ratios via freeze-drying and underwent antibacterial tests against *A. actinomycetemcomitans* and *P. gingivalis*, using the plate count method. Cytotoxicity tests were performed on HGF cells, including negative control, positive control, BAM-HA (4:1), and BAM-HA (4:2) groups, with statistical analysis conducted using One-Way ANOVA and Post Hoc Bonferroni and Tukey tests. **Results:** Antibacterial tests against *A. actinomycetemcomitans* revealed significant reduction in colony count with BAM-HA ratios 4:1 (129.0 ± 12.7 CFU/mL) and 4:2 (77.3 ± 15.5 CFU/mL) compared to the negative control (186.6 ± 27.5 CFU/mL). Similar reductions were observed for *P. gingivalis*, with BAM-HA ratios 4:1 (51.3 ± 6.6 CFU/mL) and 4:2 (3.1 ± 1.5 CFU/mL) compared to the negative control (117.3 ± 22.0 CFU/mL). Cytotoxicity tests showed no significant differences in HGF cell viability and IC50 values between the negative control and BAM-HA (4:1) or BAM-HA (4:2) groups. **Conclusion:** The BAM - HA biocomposite shows antibacterial effects against *A. actinomycetemcomitans* and *P. gingivalis*. Moreover, BAM - HA ratios of 4:1 and 4:2 do not induce cytotoxic effects on human gingival fibroblasts, suggesting potential biocompatibility for clinical applications.

KEYWORDS

A. actinomycetemcomitans, antibacterial effects, BAM-HA biocomposite, cytotoxicity, *P. gingivalis*

INTRODUCTION

Tooth extraction leads to alveolar bone resorption within the first six months post-extraction causing structural changes in vertical and horizontal dimensions.[1] Alveolar bone loss during the initial three months post-extraction reaches 3.87 mm horizontally and 1.67 mm vertically.[2] Alveolar bone resorption poses challenges during the application of dental restorations and implant placement, impacting aesthetics.[3,4] Therefore, it is necessary to take action to prevent the

trauma caused by bone resorption during extraction through socket preservation. Socket preservation is a procedure intended to maintain bone volume after tooth extraction.[3] Socket preservation may involve application of materials like Bovine Amniotic Membrane (BAM). BAM can be found in bovine placentas, and shares a chemical composition resembling the human bone.[5,6] With its potential for tissue repair and regeneration, BAM accelerates re-epithelialization and wound healing. BAM exhibits osteoinductive properties and antibacterial effects due to antimicrobial peptides such as defensin, elafin, and SLPI (Secretory Leukocyte Protease Inhibitor).[7,8] Furthermore, BAM has been proven to accelerate epithelialization, possesses strong anti-inflammatory, anti-angiogenic, and analgesic effects. The application of BAM is expected to reduce alveolar bone resorption.[9]

Combining amniotic membrane and osteoconductive bone-forming material will increase bone regeneration. BAM can be combined with hydroxyapatite (HA) in the form of $Ca_{10}(PO_4)_6(OH)_2$.[10] The biocomposite of BAM-HA has shown promise in preserving bone volume.[5] HA is considered as the most stable calcium phosphate salt that contains carbonates (CO_2^{-3}), sodium (Na^+), magnesium (Mg^{2+}), iron (Fe^{2+}), fluoride (F^-), silicates, and chlorides (Cl^-). HA is extensively utilized as a biomaterial in bone tissue replacement and repair due to its excellent osteoconductive properties, lack of toxicity, and favorable biocompatibility.[11] An in vivo study found that the biocomposite combination of HA and collagen, when implanted into the bone substance, is absorbed by osteoclasts through phagocytosis.[12]

In this study, BAM and HA were combined in ratios of 4:1 and 4:2, forming a novel biocomposite sponge. This material was made in ratios 4:1 and 4:2 to enhance the properties of the combination material from previous research and also to align with existing commercial products, thereby improving its ability to prevent alveolar bone resorption. This combination aimed to maximize the individual functions of BAM and HA.[13] Unlike commercial materials using porcine collagen, BAM-HA biocomposite serves as a halal alternative for socket preservation, supporting alveolar bone regeneration.[14] BAM-HA biocomposite is not only beneficial for bone regeneration, but also plays a crucial role in healing post-extraction wounds, which involve alveolar bone, periodontal ligaments, and gingiva.[15] One inhibiting factor in this healing process is infection, leading to bacterial colonization and increased inflammation in periodontal ligaments and cementum.[15,16]

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are types of bacteria present in the oral cavity that influence the post-tooth extraction healing process. *A. actinomycetemcomitans* and *P. gingivalis*, both are found in subgingival pockets.[17] A. *actinomycetemcomitans* is a gram-negative, facultatively anaerobic, and non-motile bacterium.[18] *P. gingivalis* is a gram-negative, non-motile, and obligate anaerobic bacterium.[19] Their overabundance increases osteoclastic activity, decreases osteoblastic activity, and slows healing processes.[20] Therefore, it is necessary to conduct antibacterial tests to prevent those bacteria to worsen the alveolar bone resorption process.

In addition to its antibacterial properties, BAM-HA must exhibit non-toxicity towards human gingival fibroblasts (HGF), which is essential for wound healing.[21] Since the toxic exposure to fibroblasts can induce apoptosis or necrosis, HGF play a crucial role in responding to oral pathogens, experiencing excessive apoptosis during inflammatory conditions, and possessing the ability to initiate inflammatory processes. As a result, they contribute to the healing of gingival tissue damage during post-tooth extraction recovery processes. HGF, crucial for responding to oral pathogens, might experience increased apoptosis during inflammation, leading to tissue damage.[22]

The combination of BAM-HA is expected to become a good composition for possessing antibacterial properties, potentially accelerating bone resorption. Additionally, due to its chemical composition and inherent qualities, BAM-HA is

expected to be antibacterial and free from cytotoxic effects on HGF. As a result, any BAM-HA material designed for oral applications should ensure safety across various oral tissues, including mucosa, gingiva, pulp, and bone. Moreover, it is essential to do cytotoxicity testing to assess potential risks and guarantee biocompatibility. Based on the description provided, the aim of this study was to evaluate the antibacterial effects of BAM-HA biocomposite (ratios 4:1 and 4:2) on *A. actinomycetemcomitans* and *P. gingivalis* while analyzing the cytotoxic effects on HGF.

In contrast to earlier research, this study prioritized the safety assessment of BAM-HA across various oral tissues, integrating thorough antibacterial effects and cytotoxicity testing to ensure biocompatibility. The study also emphasized the necessity for materials used in the oral cavity to be safe for all oral tissues, including mucosa, gingiva, pulp, and bone, without containing soluble toxic substances that could enter the bloodstream and induce systemic toxic responses. The aim of the study was to analyzed both the antibacterial effects and cytotoxicity of the BAM-HA biocomposite to ensure its suitability for clinical use and biocompatibility of the BAM-HA biocomposite before its clinical application.

METHODS

This study was in vitro experimental laboratory design utilizing the post-test-only control group design in vitro. The process began by cleaning fresh BAM from blood clots and washing it four times for 10 minutes using a 0.05% saline solution. The BAM was further washed with aquadest until the saline solution was clear, then cut into pieces and mixed with NaCl in a 1:1 ratio. This mixture was then homogenized into amnion porridge. In order to create BAM-HA biocomposite at a 4:1 ratio, 20 mL of amnion porridge was mixed with 5 mL of HA powder. The 4:2 ratio combined 20 mL of amnion porridge with 10 mL of HA powder.

The resulting mixture was homogenized, placed in a 10 cm diameter container, and frozen at -80°C for 24 hours. Subsequently, freeze-drying was performed for 48 hours at 100°C. The BAM-HA combination was sterilized through a 25 kGy gamma irradiation and stored in conical tubes at a low temperature, around 2°C-8°C.

The study samples included BAM-HA in sponge form with ratios of 4:1 and 4:2 and Bio-Oss Collagen. Additionally, pure cultures of A. actinomycetemcomitans ATCC 29522 and P. gingivalis ATCC 33277 from MiCORE Laboratory stock were used. BAM-HA, at a quantity of 100 µg, was applied to human gingival fibroblasts (HGF) at a density of 1 x 104 cells/well. In the antibacterial testing, BAM-HA and Bio-Oss Collagen (Geistlich, UK) sponge samples were cut into circles with a 5 mm diameter and sterilized with 25 kGy gamma radiation. Bio-Oss collagen was used as a positive control due to its popularity as a socket preservation material. This material has the same structure with BAM-HA biocomposite and has been proven to have a good capability in bone regeneration.

The culture media for both bacteria were prepared using BHI-B medium by dissolving 3.7 grams of BHI-B powder in 100 mL of sterile distilled water in an Erlenmeyer flask covered with aluminum foil. The mixture was autoclaved at 121°C for 15 minutes to achieve homogeneity and sterility. Subsequently, *A. actinomycetemcomitans* ATCC 29522 and *P. gingivalis* ATCC 33277 stocks from the MiCORE Laboratory were drawn using sterile needles and introduced into the broth. Both bacteria were homogenized with a vortex, and the solution was incubated in an anaerobic jar (Oxoid) at 37°C for 24 hours. Additionally, BHI-A medium (Brain Heart Infusion Agar, Oxoid) was prepared by dissolving 9.25 grams of BHI-B powder (Oxoid) and 3.75 grams of bacteriological agar powder (Himedia) in 250 mL of sterile distilled water in an Erlenmeyer flask.[23]

After sterilization at 121°C for 15 minutes, the solution was poured into sterile petri dishes (4 mm thickness) and allowed to solidify.[24] Bacterial cultures were then prepared in 10 mL of sterile PBS solution with turbidity adjusted to

McFarland standard 0.5, corresponding to 1.5x10^8 CFU/mL. The obtained bacterial cultures were diluted tenfold, and 375 µL of bacterial culture was mixed with 1125 µL of BHI-B medium in a microplate for each group.

Bacterial suspensions were added to microplate wells for each sample group. After 24 hours of incubation at 37°C, 4 µL of each sample was taken, diluted 1,000,000 times with sterile PBS, and streaked onto agar plates. The plates were incubated r an additional 24 hours at 37°C. Antibacterial activity was assessed based on the total plate count and CFU/mL.[25] Data were processed using Statistical Product and Service Solution (SPSS), with normality tested using the Shapiro-Wilk test. If normality was confirmed, one-way ANOVA was conducted, followed by post hoc testing using Bonferroni if $p<0.05$.

For cytotoxicity testing, BAM-HA biocomposite was extracted from its storage media (conical tube). Subsequently, the material was cut using a surgical knife, and its weight was accurately measured using a digital scale. The desired weight for each well was set at 100 µg of the BAM-HA sample.

Human Gingival Fibroblast (HGF) cells underwent cell culture processes. HGF cells were propagated in dishes containing 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. The incubation took place at 37°C in a humidified environment with 5% CO_2. Every two days, the cell culture medium was replaced, and the cells reached optimal density after 7 days. Cells were rinsed with Hank's solution and incubated with trypsin for 4 minutes. A mixture of 5 mL DMEM and FBS was added beneath the cell monolayer to facilitate cell detachment. Homogenized HGF cells were then placed in a 96-well microplate at a density of 2 x 10^5 cells/mL, and incubated for 24 hours.[26]

HGF cells were harvested when reaching approximately 80% cell density. The harvest involved removing the HGF cell culture medium from the CO_2 incubator, followed by media disposal using a micropipette. Next, 3 mL of phosphate-buffered saline (PBS) was added to the cell culture, and the solution was incubated. The PBS solution was then aspirated with a micropipette and discarded. Subsequently, 3 mL of trypsin solution was introduced into the cell culture medium, followed by a 5-minute incubation in the CO_2 incubator. Afterward, cells were examined under a microscope to evaluate their condition. The subsequent steps included inactivation, resuspension, and observation under a microscope using PGS, trypan blue, and the suspended cell solution.[27,28]

The study comprised four groups: negative control group, positive control group, and two treatment groups. The negative control group consisted of untreated HGF, the positive control group involved 0.1 mg of Bio-Oss Collagen, and the treatment groups were treated with either BAM-HA composite in a 4:1 or 4:2 ratio. In the negative control group, 10,000 cells/well of HGF were placed in a 96-well microplate, while in the positive control group, 0.1 mg of Bio-Oss Collagen was applied to a 96-well microplate containing 10,000 cells/well of HGF. In the BAM-HA 4:1 group, treatment included 10,000 cells/well of HGF and 0.1 mg of BAM-HA composite in a 4:1 ratio in a 96-well microplate. Similarly, in the BAM-HA 4:2 group, treatment involved 10,000 cells/well of HGF and 0.1 mg of BAM-HA composite in a 4:2 ratio in a 96-well microplate. Each treatment group was replicated seven times, and the microplate was placed in an incubator at 37°C for 24 hours.

Subsequently, the cell growth medium was removed, and washing was performed using 100 µl of phosphate-buffered saline (PBS). Then, 100 µL of a solution containing 10 µL of CCK-8 with 90 µL of PBS was added to each well. The microplate was incubated for approximately 4 hours at 37°C and mechanically stirred using a plate shaker for 5 minutes to ensure complete dissolution of formazan crystals. Further washing with Dimethyl sulfoxide (DMSO) was carried out. Living HGF cells were stained with formazan, resulting in an orange color, while dead cells did not show orange coloration. Formazan absorbance was read using a 96-well Microplate reader at a wavelength of 450 nm to obtain Optical

Density (OD) values. The more intense the formazan color, the higher the absorbance value, indicating a higher number of viable cells.[29]

The obtained data were compared with ISO 10993-5 standards. According to ISO 10993-5, cell viability percentages above 80% are considered non-cytotoxic; 80% - 60% indicates low cytotoxicity; 60% - 40% suggests moderate cytotoxicity, and below 40% indicates high cytotoxicity.[30] A higher IC50 value corresponds to lower material toxicity. The acquired data were analyzed using the Kolmogorov-Smirnov normality test, Levene's test for variance homogeneity, and One-way ANOVA analysis with a significance level of $p<\alpha$ ($\alpha=0.05$). In case of differences, post-hoc Tukey tests were conducted.

RESULTS

The antibacterial analysis of *A. actinomycetemcomitans* revealed round white colonies on the agar medium. The petri dish was divided into three sections for repetitions, and bacterial colony counting was performed in each section. The negative control group exhibited more bacteria than other groups (Figure 1A). The BAM-HA 4:1 group showed more rounded shapes than the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups (Figure 1B). The BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups had similar quantities of rounded shapes (Figure 1C, 1D).

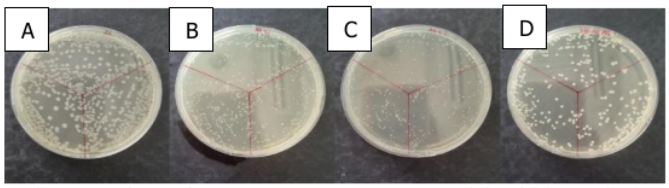
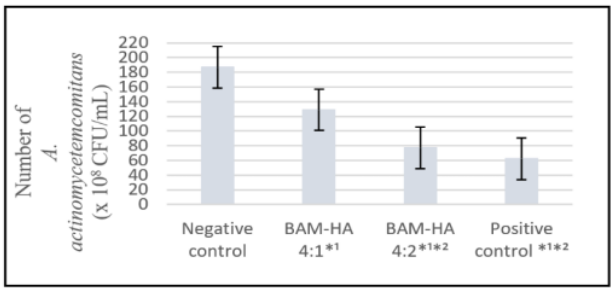


Figure 1. illustrates colonies of A. actinomycetemcomitans in Petri dishes (A) bacterial colonies without treatment (negative control), (B) bacterial colonies after treatment with BAM-HA biocomposite at a 4:1 ratio, (C) bacterial colonies after treatment with BAM-HA biocomposite at a 4:2 ratio, and (D) bacterial colonies after treatment with Bio-Oss Collagen (positive control).

The average bacterial colony counts in the four test groups were (186.6 ± 27.5)x10^8 CFU/mL, (129.0±12.7)x10^8 CFU/mL, (77.3±15.5)x10^8 CFU/mL, and (62.3±2.5)x10^8 CFU/mL, respectively (Graphics 1). The percentage reduction in colony count compared to the negative control for the three test groups was 30.8, 58.5, and 66.6%, respectively.



Graphics 1. Average colony of *A. actinomycetemcomitans* in antibacterial testing
*1 There were significant differences with negative control groups
*2 There were significant differences with BAM-HA 4:1 groups

Statistical analysis using one-way ANOVA and post hoc Bonferroni tests indicated significant differences between the negative control and BAM-HA 4:1, 4:2, and Bio-Oss Collagen (positive control) groups, with $p < 0.05$. The BAM-HA 4:1 group also showed a significant difference ($p < 0.05$) compared to the BAM-

HA 4:2 and Bio-Oss Collagen (positive control) groups. However, the BAM-HA 4:2 group did not exhibit significant differences from the Bio-Oss Collagen (positive control) group, with a p-value of 1 (Graphics 1 & Table 1).

Table 1. Statistical Analysis of one-way ANOVA with post-hoc Bonferroni Test for the Antibacterial Effect of BAM-HA Biocomposite on *A. actinomycetemcomitans*

Control Group	Negative Control	BAM-HA 4:1	BAM-HA 4:2	Positive Control
Negative		0.020*	0.001*	0.001*
BAM-HA 4:1			0.036*	0.008*
BAM-HA 4:2				1
Positive				

In the antibacterial test against *P. gingivalis*, observations revealed rounded white colonies on the agar medium. The negative control group exhibited more bacteria than other groups (Figure 2A). The BAM-HA 4:1 group showed significantly more rounded shapes than the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups (Figure 2B). The BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups had minimal and almost equal quantities of bacteria (Figure 2C, 2D).

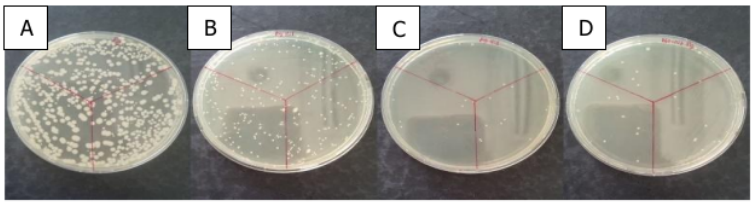
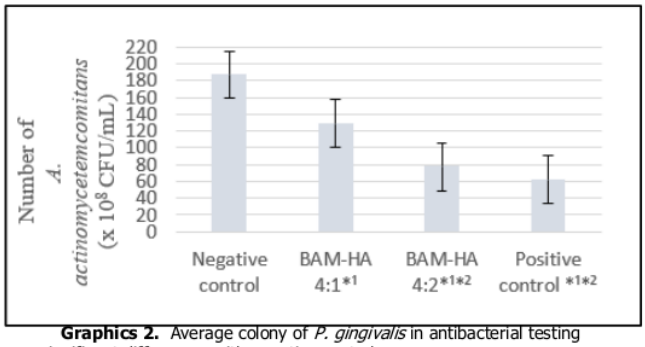


Figure 2. Colonies of *P. gingivalis* in Petri dishes (A) bacterial colonies without treatment (negative control) (B) bacterial colonies after treatment with BAM-HA biocomposite 4:1 (C) bacterial colonies after treatment with BAM-HA biocomposite 4:2 (D) bacterial colonies after treatment with Bio-Oss Collagen (positive control)

The average bacterial colony counts in the four test groups were (117.3±22.0) x 10^8 CFU/mL, (51.3± 6.6) x 10^8 CFU/mL, (3.1±1.5) x 10^8 CFU/mL, and (4.5±1.3)x10^8 CFU/mL, respectively (Table 4). The percentage reduction in colony count compared to the negative control for the three test groups was 56.2%, 97.3%, and 96.1%, respectively (Graphics 2).

Statistical analysis using one-way ANOVA and post hoc Bonferroni tests indicated significant differences ($p<0.05$) between the negative control with BAM-HA 4:1, 4:2, and Bio-Oss Collagen (positive control) groups. The BAM-HA 4:1 group also exhibited a significant difference ($p<0.05$) compared to the BAM-HA 4:2 and Bio-Oss Collagen (positive control) groups. However, the BAM-HA 4:2 group did not differ significantly from the Bio-Oss Collagen (positive control) group, with a p-value of 1 (Graphics 2 & Table 2).

For the Cytotoxicity Test, during the research, two visual observations of HGF were conducted under a microscope. The first observation was made before treatment with Bio-Oss Collagen, BAM-HA ratio 4:1, and BAM-HA ratio 4:2 on HGF. This observation indicated the success of cell culture and suitable conditions for proceeding to the following research phase.



Graphics 2. Average colony of *P. gingivalis* in antibacterial testing
*1 There were significant differences with negative control groups
*2 There were significant differences with BAM-HA 4:1 groups

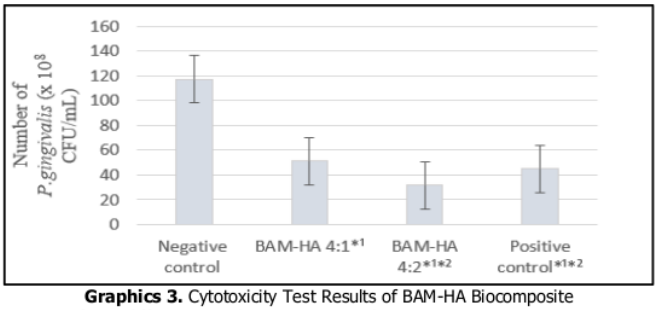
Table 2. Statistical Analysis of one-way ANOVA with post-hoc Bonferroni Test for the Antibacterial Effect of BAM-HA Biocomposite on P. gingivalis

Control Group	Negative Control	BAM-HA 4:1	BAM-HA 4:2	Positive Control
Negative		0.001*	0.001*	0.001*
BAM-HA 4:1			0.006*	0.007*
BAM-HA 4:2				1
Positive				

Subsequent observations were made after cells were treated with Bio-Oss Collagen, BAM-HA ratio 4:1, BAM-HA ratio 4:2, and incubated for 24 hours before CCK-8 administration. This observation reflected the initial step in evaluating cell responses to treatment before proceeding to the next step, CCK-8 testing and cell viability analysis to understand the toxic effects of these materials on HGF more deeply.

Visual observations of cell viability continued after applying the CCK-8 reagent and subsequent one-hour incubation. Further identification of the color change, reflecting the activity of live cell dehydrogenase enzymes, was more accurately performed using a microplate reader. The results obtained were optical density (OD) values.

The Optical Density (OD) values from the cytotoxicity test of BAM-HA ratio 4:1, BAM-HA ratio 4:2, and Bio-Oss Collagen on HGF were then converted using formulas for cell viability percentage and cell inhibition percentage. Cell viability and IC50 values from each test group were obtained (Graphics 3).



Graphics 3. Cytotoxicity Test Results of BAM-HA Biocomposite
* There were significant differences with negative control groups

Graphics 3 illustrates the average and standard deviation values of cell viability in various groups. The cell viability value for the negative control group was 100±3.35, while the positive control group had a value of 79.30±4.92. In the BAM-HA 4:1 and 4:2 treatment groups, cell viabilities reached 80.63±3.28 and 90.69±3.28. Graphics 3 also provides information on the IC50 values for each test

group: positive control group (78.20), BAM-HA 4:1 treatment group (81.91), and BAM-HA 4:2 treatment group (89.58).

Statistical analysis indicated normal data distribution ($p>0.05$) based on the Kolmogorov-Smirnov normality test for all sample groups. The homogeneity of variance test using Levene's test showed homogeneous variance data ($p>0.05$) with a p-value of 0.362. One-way ANOVA resulted in a significance of 0.0001 ($p<0.05$), indicating a significant difference in the average cell viability values among sample groups.

The post-hoc Bonferroni test showed a significant difference in cell viability values between the negative and positive control groups. There was no significant difference in cell viability values between the control group and BAM-HA 4:1 and 4:2 groups, indicating no significant difference in cell viability among these three groups. The same applied to cell viability values between the Bio-Oss Collagen group and the BAM-HA 4:1 and 4:2 groups.

DISCUSSION

Based on the antibacterial tests conducted on samples, there was a decrease in the quantity of *A. actinomycetemcomitans* and *P. gingivalis* bacteria in the BAM-HA 4:1 and 4:2 (Graphics 1 and Graphics 2). These findings were consistent with the previous research where bone scaffold materials with hydroxyapatite as the main component exhibited antibacterial effects against gram-negative bacteria.[31] Other research by Cunniffe et al also indicated that hydroxyapatite exhibits antibacterial properties.[32] Furthermore, the reduction percentage in the quantity of *P. gingivalis* bacteria in BAM-HA 4:1 and 4:2 biocomposites was more significant compared to *A. actinomycetemcomitans* bacteria. This difference is due to variations in the composition of cell wall structures and lipopolysaccharides (LSP) in each bacterium, causing different sensitivities to the biocomposite.[33]

This study also showed that applying Bio-Oss Collagen material can reduce the growth of both *A. actinomycetemcomitans* and *P. gingivalis* bacteria. Bio-Oss Collagen is widely used in socket preservation and consists of 90% deproteinized bovine bone mineral (DBBM) and 10% collagen. The main component of DBBM is hydroxyapatite, indicating similar antibacterial mechanisms to the BAM-HA biocomposite.[34] Therefore, the antibacterial test results suggest that the BAM-HA biocomposite has capabilities comparable to Bio-Oss Collagen in reducing bacterial growth.

Furthermore, regarding cytotoxicity, this study found that cell viability in the negative control group reached 100%. Correspondingly, findings from a study by Neto et al.[35] exhibited comparable results, indicating 100% viability in the negative control cells group. This viability value was obtained in the cytotoxicity test using the CCK-8 Assay method, reflecting the cell survival after treatment. Cells in this group were not exposed to foreign substances that could affect their metabolism and survival.[36] Consequently, mitochondrial dehydrogenase enzymes in the cells reduced the CCK-8 reagent (WST-8) to form orange-colored formazan. The formazan concentration values indicated perfect cell viability. The negative control group did not have an IC_{50} value because cells in this group were not exposed to any foreign substances that could cause a decrease in the number of living cells, making cell viability the reference baseline or normal condition that does not require IC_{50} calculation.

The viability values for the BAM-HA 4:1 and 4:2 treatment groups, as indicated in Graphics 3, were 80.6% and 90.6%, with concomitant elevated IC50 values standing at 81.91 and 89.58. This result is in concordance with a study by Octarina et al.,[37] wherein a significantly high fibroblast viability of 98.14% was observed for BAM-HA with a 35:65 ratio. Lower cytotoxicity levels can be associated with high IC_{50} values. It means that the biomaterial has minimal negative impact on the cells' survival ability. The cytotoxicity test results in the positive control group showed a cell viability value lower than the treatment

groups, at 79.3%. Consistent with previous research, it is known that the viability of Bio-Oss Collagen cells gradually decreases.[38] The gradual decrease in cell viability, indicating cell death due to exposure to foreign substances, can be triggered by various factors, one of which is related to the content of the biomaterial. Therefore, using biomaterial with a specific viability percentage should be limited in certain exposures to remain within the body's tolerance range. This research was confined to laboratory investigations. Further studies targeting osteoblast cells and animals are essential to validate BAM-HA biocomposite efficacy in preventing alveolar bone resorption.

CONCLUSION

The BAM-HA biocomposite has antibacterial effects on both *A.actinomycetemcomitans* and *P. gingivalis* bacteria. The biocomposite with ratios of 4:1 and 4:2 exhibits antibacterial effects against both bacteria. This study also shows that BAM-HA ratios of 4:1 and 4:2 do not have cytotoxic effects on human gingival fibroblasts. However, further supporting tests are required, including in vivo studies on animal models and clinical trials to assess the effects of the BAM-HA biocomposite. Additionally, further in vitro research involving osteoblasts is recommended, followed by experiments on animals and clinical trials to verify the biocompatibility and impacts of the biomaterial. Implications of this research: it is hoped that this material can prevent alveolar bone resorption after extraction.

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Informed Consent Statement: Not applicable.
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