ORIGINAL ARTICLE

Enhancing Bovine Hydroxyapatite: The Role of Crosslinker and Secretome in Biomechanical Properties and Toxicity

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ABSTRACT

Background: Advancements in biomaterials aid in hard tissue repair. Hydroxyapatite (HAP) similar to natural bone and used in bone grafts. Crosslinking agents like poly-vinyl alcohol (PVA) and glutaraldehyde (GTA), combined with bone marrow-derived mesenchymal stem cell (BM-MSC) secretome, are being explored for their potential to enhance HAP's biomechanical properties and minimize toxicity. This study investigates how crosslinkers and secretomes affect the toxicity and biomechanical characteristics of bovine hydroxyapatite (BHA). **Methods:** This in vitro laboratory study employed a comparative design. HAP was crosslinked with PVA at a 0.5% concentration and with GTA at 0.1% and 1% concentrations, both with and without BM-MSC secretome. Mechanical properties, including compressive and bending strength, were measured. Toxicity levels and water content were also analyzed using MTT Assay. Data were analyzed descriptively and statistically using ANOVA dan Tukey's Method for Post Hoc Analysis. **Results:** Addition of crosslinker and secretome significantly impacted bending strength ($p=0.004$), but not compressive strength ($p=0.648$). The HAP + PVA 0.5% + secretome group had the highest compressive and bending strength compared to the control, HAP + GTA 0.1% + secretome, and HAP + GTA 1% + secretome groups. While addition crosslinker and secretome tended to reduce HAP toxicity and water content, differences were not statistically significant ($p > 0.05$ and $p > 0.05$, respectively). **Conclusion:** The biomechanical properties of HAP are improved by the addition of 0.5% PVA, to enhanced compressive and bending strengths. GTA's effectiveness is concentration-dependent, with higher concentrations causing toxicity. Determining optimal concentration is crucial for safe clinical application.
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INTRODUCTION

Bone defects, defined as the reduction of bone mass, are often caused by congenital abnormalities, tumors, and other diseases. These defects can range from minor or massive, with the latter characterized by gaps exceedingly twice the diameter of the bone. For large gaps, bone grafting—a technique that uses material to enhance bone regeneration—is often employed (1).

Hydroxyapatite (HAP) is a biomaterial that has garnered attention due to its thermodynamic stability in bodily fluids. HAP is biocompatible and bioactive because its chemical structure closely resembles the mineral content of human bones. It also possesses excellent mechanical strength, porosity, osteoconductivity, osteoinductivity, and osteointegration. The osteoconductive properties of HAP are crucial in bone regeneration, stimulating mesenchymal cell proliferation and differentiation. HAP is used in orthopedics, maxillofacial implants, and dentistry to repair or replace tissues. Studies show that HAP extracted from bovine bone exhibits great strength, emphasizing the need for comprehensive testing (2).

However, HAP is brittle, difficult to manipulate, and remains stable only at the implant site. Synthetic HAP is also brittle and has low strength due to differences in microstructure and composition from human bone, leading to prolonged healing. To address this brittleness, research has focused on developing coatings and composite materials to reduce structural fragility through cross-linking (3).

Cross-linking involves forming covalent bonds between polymer chains, which can be achieved using synthetic or natural monomers or polymers, either physically or chemically. Chemical cross-linking agents like glutaraldehyde (GA), which is highly soluble in various solvents, and polyvinyl alcohol (PVA), known for its inert and stable nature, are commonly used. Additionally,

bone marrow-derived mesenchymal stem cells (BM-MSCs) produce bioactive factors crucial for tissue repair and regeneration through paracrine signaling (4). Bone marrow-derived mesenchymal stem cells are utilized as an adjunct therapy to enhance the properties of hydroxyapatite (HAP) when crosslinked with specific agents such as PVA and glutaraldehyde (GTA). Rather than being the primary treatment modality, BM-MSCs contribute through their secretome, which includes bioactive factors crucial for tissue repair and regeneration. This adjunctive role allows the BM-MSC secretome to complement HAP's structural support by leveraging paracrine signaling to aid in cellular processes like osteoconduction and osteoinduction, critical for bone regeneration. The secretome's components support rapid angiogenesis, which is critical for bone healing by enhancing osteoclast activity, increasing osteoblast function, and facilitating the formation of new blood vessels. Studies have shown that mesenchymal stem cells (MSC) secretomes accelerate these regenerative processes, aiding in quicker and more effective bone formation. The biomechanical properties of grafts should ideally match those of normal bone. Compressive strength is commonly used to measure these properties, essential for determining biomaterial strength and elasticity before human application. Biomaterials must also undergo toxicity and cell viability testing. The MTT assay evaluates toxicity and provides indirect information about cell viability. Glutaraldehyde is efficient and widely used for collagen materials, with numerous bioprosthetic implants indicating its clinical acceptability despite some cytotoxicity reports. PVA is biocompatible and forms hydrogels, showing promising results in artificial cartilage development. PVA/HA composites possess good biocompatibility, bioactivity, mechanical strength, and low cytotoxicity (5). PVA-enriched HAP composites improve bioactivity and cytocompatibility, significantly enhancing the potential for bone regeneration in vivo (6). Similarly, Ashraf et al. showed that adding PVA to HAP scaffolds enhances porosity while maintaining structural integrity, crucial for cell infiltration and vascularization, which are necessary for successful bone healing (7). Studies by Nguyen et al. explored PVA's role in increasing the compressive modulus in bone scaffolds, revealing that PVA crosslinking can significantly strengthen the HAP matrix and reduce brittleness, a major limitation of pure HAP. This improvement is attributed to hydrogen bonding between PVA and HAP, contributing to the overall flexibility and robustness of the scaffold (8). Kamarul et al. further confirmed PVA's biocompatibility in rat models, demonstrating no inflammatory response, thus supporting PVA's safe application in biomedical contexts (9).

GTA is a potent crosslinker commonly used in HAP composite materials for its ability to create strong intermolecular bonds within the polymer matrix, thereby enhancing the stiffness and durability of the composite. Studies by Azami et al. revealed that GTA crosslinking increases Young's modulus in HAP composites, effectively enhancing the structural resilience of the material at concentrations around 0.25-1%. However, higher concentrations can induce cytotoxicity, as excessive crosslinking may impair biocompatibility, underscoring the need for optimized dosages (10). GTA crosslinking improves the bending strength of HAP scaffolds, providing critical mechanical support in load-bearing environments while maintaining bioactivity when applied at lower concentrations. Moreover, Wu et al. found that GTA-crosslinked HAP scaffolds show reduced biodegradation rates, which allows the composite to retain its structural form longer, supporting prolonged bone regeneration (5). Additionally, GTA's effect on pore size and connectivity, indicating that optimized GTA crosslinking can create favorable microenvironments for osteoblast adhesion and growth, aiding in successful bone integration. Both PVA and GTA contribute to enhancing HAP's mechanical and biological properties. PVA provides flexibility and improved compressive strength, making it ideal for creating load-bearing composites with high biocompatibility. In contrast, GTA's impact on HAP lies in increasing rigidity and durability through strong crosslinking, making it suitable for applications where prolonged scaffold stability is critical, albeit with careful control of concentration to mitigate cytotoxic risks (11). Based on the above information, cross-linking HAP with other biomaterials is crucial and must be tested thoroughly before human application. The main goal of this study is to investigate how crosslinkers and secretomes affect the toxicity and biomechanical characteristics of bovine hydroxyapatite (BHA). This study addresses these gaps by systematically evaluating the influence of different PVA and GTA concentrations on HAP's biomechanical properties and cytocompatibility. By incorporating BM-MSC secretome as an adjunct, this research uniquely combines mechanical strengthening with regenerative enhancement, potentially creating a composite with both structural robustness and bioactivity. Through comprehensive testing—including compressive and bending strength, toxicity, and water content—this study aims to establish a balanced, optimized crosslinker approach. Thus, this research not only fills the existing knowledge gap on crosslinker optimization in HAP composites but also provides a practical solution for developing safer, stronger, and more bioactive scaffolds suitable for clinical bone repair.

MATERIAL AND METHODS

HAP Production and Sampling

HAP and BM-MSC secretome were prepared at the Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, while Polyvinyl Alcohol from Sigma Aldrich and Glutaraldehyde were used as cross-linking agents. The HAP and BM-MSC were obtained from human donors

who provided informed consent and informed to consent. The sample size was calculated using Federer's formula (1963), resulting in a total of 25 samples, divided into 5 groups: control; HAP only; HAP + 0.5% PVA + secretome; HAP + 0.1% GTA + secretome; and HAP + 1% GTA + secretome. The second phase aimed to evaluate compression, bending strength, and toxicity.

This experimental in vitro study analyzed the compressive and bending strength of crosslinked HAP, along with toxicity levels. The data was observed descriptively and quantitatively, followed by an ANOVA test and Tukey's Method for Post Hoc Analysis. Equipment used included powder scales, an oven, an MTT assay for toxicity testing, a compression tester, and a Bell Engineering Series I ThermoG L-M for water content measurement.

The research was conducted at the Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia, from December 2020 to December 2022. Polyvinyl Alcohol (0.5%) and Glutaraldehyde (0.1% and 1%) served as crosslinkers, each with safe doses ranging from 0.1% to 2.5%.

Bovine HAP Preparation

Bovine bones were cleaned and shaped into cubic hydroxyapatite (HAP) forms. After thorough cleaning, the bones were furnaced at 1000°C, followed by freeze-drying at -80°C for 24 hours and lyophilization at -100°C for 48 hours. The resulting HAP was obtained from the Cell & Tissue Bank Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

Crosslinking Process

Sterilized bovine HAP was divided into two groups and crosslinked with 0.5% PVA and 0.1% or 1% GA. The process was conducted under sterile conditions, soaking the HAP in the crosslinker solution and drying it at 120°C for 120 minutes.

Crosslinked HAP/ Secretome Preparation

The synthesis of crosslinked HAP begins by immersing HAP in a crosslinker solution (L-Glutamic Dehydrogenase) and drying it using Dehydrothermal Treatment (DHT). Next, the HAP is combined with BM-MSC and preserved through freeze-drying. The final product is then drained in a Biological Safety Cabinet (BSC).

Compression and Bending Strength Measurement

Each HAP group, with nine replicates, was tested for compression and bending strength using an autograft machine at Laboratory of Science and Technology, Faculty of Science and Technology, Universitas Airlangga. The HAP used measured 3x1x1 cm for bending tests and 1x1 cm for compression tests. Compression tests applied vertical pressure at one point while bending tests applied pressure at three points. The results were represented as Young's Modulus in MPa (Figure 1).

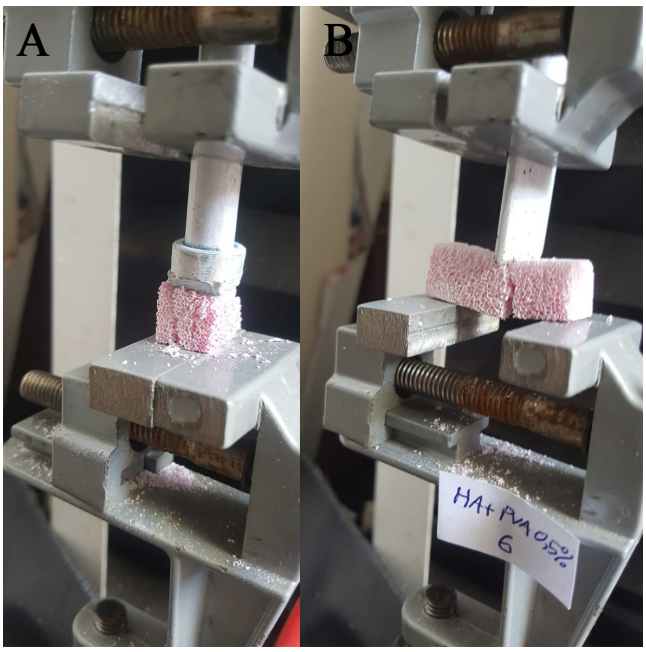

Figure 1: A. Compression Test; B. Bending Test

MTT Assay for Toxicity Testing

Crosslinked HAP was placed in well plates containing BM-MSC secretome and incubated at 37°C with 5% CO2 for 24 hours. After adding 10 µL of 5 mg/ml MTT solution, the plates were incubated for another 4 hours. Sodium dodecyl sulfate (SDS) stopper reagent was then added, and the plates were left for 24 hours before measuring absorbance at 595 nm with a microplate reader.

Water Content Testing

Samples weighing 0.28 ± 0.05 g were wrapped in paper, placed on a moisture balance, and dried. The drying mode measured the temperature, and the sample was evenly spread on the sample plate. Results were obtained in 1-2 minutes. After three replicates, the device was rested for 25 minutes before the next measurement.

Statistical Analysis

For this study, descriptive statistics summarized the mean and standard deviation for each group's compressive and bending strength, toxicity, and water content. A one-way ANOVA tested for statistical significance across groups with different concentrations and combinations of cross-linkers (PVA and GTA) and secretomes. Statistical significance was set at $p < 0.05$. When ANOVA results were significant, post-hoc tests identified specific group differences. This analysis provided insights into the biomechanical and toxicity effects of each treatment, helping determine optimal concentrations and cross-linker types for enhancing bovine hydroxyapatite (BHA) stability and biocompatibility. The data were analyzed using descriptive and quantitative methods with the assistance of SPSS software version 21.0

(IBM Corporation, Armonk, NY, USA). The results are presented in the form of tables and graphs for clear visualization.

RESULTS

This study evaluates the effects of various cross-linking agents on the mechanical properties, toxicity, and water content of HAP-based biomaterials. It tests the compression strength, bending strength, toxicity levels, and moisture balance of five different formulations: Control; HAP only, HAP + 0.5% PVA + secretome; HAP + 0.1% GTA + Secretome; and HAP + 1% GTA + secretome.

The compressive strength varied across treatment groups. The HAP group without crosslinking achieved a mean compressive strength of 0.5378 ± 0.067 MPa, while the HAP + 0.5% PVA group had a slightly higher mean of 0.5911 ± 0.0741 MPa. The HAP + 0.1% GTA group showed a mean compressive strength of 0.5044 ± 0.0427 MPa, and the HAP + 1% GTA group had a mean of 0.4700 ± 0.007 MPa (Table I).

Table I: Compression Test Results

No	Items	Compression Test (MPa)									Mean (± SD)
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	
1	HA	0,092	0,67	0,55	0,43	0,32	0,64	0,63	0,32	0,36	0, 5378 ± 0,067
2	HA + PVA 0,5% + *Secretome*	0,43	0,76	0,61	1	0,51	0,42	0,37	0,82	0,4	0,5911 ± 0,0741
3	HA + GTA 0,1% + *Secretome*	0,48	0,54	0,55	0,41	0,43	0,63	0,71	0,52	0,27	0,5044 ± 0,0427
4	HA + GTA 1% + *Secretome*	0,78	0,5	0,67	0,78	0,25	0,22	0,38	0,34	0,31	0,4700 ± 0,007

The toxicity levels of HAP, with and without crosslinking, were assessed using the MTT assay. Toxicity levels were 98.0633% ± 1.9367% in the control group, 93.2867% ± 0.8403% in the HAP group, 86.9167% ± 2.2141% in the HAP + 0.5% PVA group, 85.5867% ± 1.8010% in the HAP + 0.1% GTA group, and 94.1800% ± 4.6671% in the HAP + 1% GTA group (Table II).

Table II: Toxicity Test Results

No	Items	Toxicity Mean (± SD)
1	Cell Control	98,0633 ± 1,9367
2	HA	93,2867 ± 0,8403
3	HA + PVA 0,5% + *Secretome*	86,9167 ± 2,2141
4	HA + GTA 0,1% + *Secretome*	85,5867 ± 1,8010
5	HA + GTA 1% + *Secretome*	94,1800 ± 4,6671

Compression tests revealed that HA with PVA 0.5% had the highest average compression strength (0.5911 MPa), though statistical tests (Kruskal-Wallis, p=0.648) indicated no significant difference across groups. In bending strength, HA with PVA 0.5% also showed superior results, with a significant difference found between groups (Kruskal-Wallis, p=0.005), particularly between HA+GTA 1% and HA+PVA 0.5% (p=0.004) (Table III). Toxicity assays, measured through MTT, showed no significant differences among the formulations (p=0.077), with HA+GTA 1% displaying slightly elevated levels (94.18%). Moisture content tests found the control group to have the highest water content, with no significant differences across groups based on Kruskal-Wallis and ANOVA tests (p=0.202).

The study concludes that while some formulations demonstrated enhanced mechanical properties, statistical significance was limited. The findings suggest a need for further exploration of cross-linking techniques to optimize biomaterial performance in orthopedic applications. ANOVA also found no significant differences in water content between or within treatment groups.

Table III: Bending Test Results

No	Items	Bending Test (MPa)									Mean (± SD)
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	
1	HA	1,16	1,6	1,52	0,48	0,35	0,07	0,14	0,13	0,46	0,6544 ± 0,2009
2	HA + PVA 0,5% + *Secretome*	1,51	1,63	1,06	1,63	0,8	0,34	1,67	0,49	0,66	1,3067 ± 0,1255
3	HA + GTA 0,1% + *Secretome*	0,19	1,31	0,67	1,48	0,67	0,76	0,07	0,28	0,57	0,6667 ± 0,1592
4	HA + GTA 1% + *Secretome*	0,09	0,72	0,29	0,76	0,77	0,5	0,04	0,03	0,45	0,4056 ± 0,1024

DISCUSSION

Hydroxyapatite is an inert bioceramic scaffold used to fill bone defects (6,12,13). Initially, HA was used as a graft that did not interact with surrounding tissues. However, it has evolved into a reactive material that can act as a conductive scaffold for bone growth. Structurally similar to bone apatite, HAP is a key inorganic component of bones and teeth (14).

Bone defects from surgeries or infections require grafts to stimulate bone growth, making HAP a potential graft material due to its regenerative properties (6,12,13). Bone typically contains 69% mineral, 22% organic matrix, and 9% water. HAP is stable and insoluble, with a calcium/phosphate ratio of 1.67. Its mechanical properties depend on porosity, density, crystal size, and composition. Bending strength ranges from 38-250 MPa, compressive strength from 120-150 MPa, and tensile strength from 38-300 MPa (15).

HAP's biocompatibility means it has no local or systemic toxicity. When implanted, it supports new bone growth, adhering directly to the implant surface (15). HAP surfaces promote osteoblast adhesion, growth, and differentiation and can bind and concentrate bone morphogenetic proteins (BMP).

HAP's interaction with biological tissues is crucial for bone regeneration, which starts from the scaffold. Advances in technology have led to hybrid materials designed to spontaneously self-organize, enhancing their scaffold properties (15). Bioactive HAP gradually dissolves, forming biological apatite before interacting with tissues, providing stability under mechanical loads. This bioabsorbable material acts as a scaffold and filling material, aiding bone healing similar to fracture repair (15).

Biodegradation of hydroxyapatite (HAP) begins with changes in surrounding biofluids and absorption of biomolecules. The biochemical dissolution depends on surface area, volume, fluid convection, acidity, and temperature (15). Dissolution inversely correlates with the Ca/P ratio, purity, crystal size, and surface area. HAP is more stable than other calcium orthophosphates like tricalcium phosphate (TCP). Osteoclasts mainly mediate bioresorption, sometimes assisted by macrophages (12). Degradation depends on the HAP/ TCP ratio, with higher ratios being less degradable (15).

Surface roughness and porosity are crucial for HAP's chemical bonding. After implantation, proteins absorb onto HAP's surface. Pore size and interconnectivity influence bone growth and blood vessel formation. (1) Pores around 50 µm aid blood vessel formation, while 200 µm pores support osteoid growth. Pores <1 µm enhance bioactivity, protein interaction, and cell attraction. Sizes 1-20 µm direct cell orientation and bone growth. Larger pores (100-1000 µm) aid mechanical reinforcement and implant aesthetics (16).

Secretome consists of factors secreted by cells into the extracellular space, including proteins, nucleic acids, lipids, and extracellular vesicles (17). Mesenchymal stem cells secrete bioactive factors like cytokines, chemokines, growth factors, and exosomes. These factors help tissue repair and regeneration via paracrine signaling. MSC secretome has anti-apoptotic activity, wound healing benefits, and promotes tissue regeneration (1).

In bone grafts, rapid angiogenesis is crucial. Secretome supports this process. Studies show that adipose-derived MSC secretome enhances angiogenesis and bone formation more effectively than bone marrow MSCs (18). This process accelerates osteoclast phagocytosis, increases osteoblast activity, and speeds up woven bone formation while reducing fibrous tissue. The highest microvascular increase was observed in week 1 across all groups. The rise in VEGF formation may be influenced by the osteoinduction of demineralized bovine bone matrix, stimulating accelerated angiogenesis (19,20).

The addition of secretome in this experiment impacts bone tissue repair and regeneration. Secretome

from bone marrow-derived mesenchymal stem cells (BM-MSC) plays a crucial role due to its paracrine properties, which mediate intercellular signaling. This signaling enhances the biomechanical properties of hydroxyapatite (HA) by promoting the stability and elasticity needed for bone grafts to mimic normal bone characteristics. The experiment reveals that combining secretome with HA and different cross-linkers, such as polyvinyl alcohol or glutaraldehyde, contributes to the compressive and bending strength of HA, supporting improved tissue regeneration and potentially reducing cytotoxicity (19,20).

Research by Arifin et al. showed that the majority of patients achieved excellent results: 80.36% were excellent, 12.5% were good, 3.57% were fair, and 3.57% were poor (11). A study by Mahyudin et al., conducted using rabbits, which yielded results similar to this study, stated that bovine hydroxyapatite provides comparable outcomes in bone healing compared to allografts (20). Research by Tsai et al. indicated that out of 33 patients using HAP bone substitutes, X-ray images showed good bone healing with an 81.8% fusion rate after 6–12 months of monitoring (21).

Arifin et al. also explained that BHA (bovine hydroxyapatite) exhibits chemical similarity to bone, excellent biocompatibility, which stimulates osteo-conduction, and its ability to integrate into bone without causing immune reactions (11). Kotobuki et al. explained that the microenvironment of HAP can provide calcium ions and alkaline ions for osteoblasts, leading to mineralization of the extracellular matrix of mesenchymal cells and secretion of ATPase. This can also encourage cells to exhibit an osteoblastic phenotype and form bone tissue (22).

Glutaraldehyde is a chemical compound highly soluble in water, alcohol, and organic solvents. It is often used as a cross-linking agent, binding proteins intermolecularly (between different proteins) and intramolecularly (within the same protein) (23). Azami et al. found that cross-linking HAP with GTA increased Young's modulus up to 1% GTA concentration, then decreased at higher concentrations. For instance, at 0.25% GTA, the elastic modulus was 45 ± 5.5 MPa, and at 1%, it was 48.3 ± 14.0 MPa (10). However, gelatin was also used in that study, unlike our study, which used GTA at 0.1% and 1.0%. The bending strength for 0.1% GTA was 0.67 MPa and 0.45 MPa for 1.0% GTA, while the compressive strength was 0.52 MPa and 0.38 MPa, respectively. Higher GTA concentrations tend to lower mechanical strength, possibly due to gelatin's absence in our study (10).

Similar to the findings in this study, where the median strength of GTA 0.1% was higher compared to GTA 1% (0.67 MPa vs 0.45 MPa), these changes in strength may also be influenced by the toxicity induced at higher concentrations of GTA. Research has shown that GTA 0.1% exhibited significantly lower toxicity with an average of 85.59%, compared to GTA 1% which had 94.18% toxicity.

PVA (0.5%) showed significantly higher bending strength than 1% GTA but not significantly different from 0.1% GTA. PVA is known for good adhesion, film formation, and biocompatibility, though high-quality PVA is expensive (24). Kikuchi et al. found that cross-linking with collagen increased with higher GTA concentrations (25). However, too much GTA reduces mechanical strength due to excessive cross-linking and increased pore size. Higher GTA concentrations also tend to increase toxicity, as seen with a significant increase in toxicity at 1% GTA compared to 0.1% (85.59% vs. 94.18%) (26).

Azami et al. reported decreased cell populations with higher GTA concentrations due to toxicity. Our study also found lower toxicity at 0.1% GTA and 0.5% PVA compared to controls, but not significantly different at 1% GTA. Cell viability and proliferation are crucial for osteoconduction, and adding cross-linkers like GTA or PVA can enhance these properties. This contrasts with the findings who reported toxicity of GTA on osteoblasts. The mechanism of this toxicity occurs through apoptosis and is dose-dependent (10).

The water content in the GTA group was higher than in the PVA group (0.449% ± 0.562% vs. 0.374% ± 0.284%), but this was not significant ($p>0.05$). Water absorption is indicated by porosity and swelling ratio. Ashraf et al. showed increased PVA porosity with added HAP, rising from 58% to 71%. PVA's use in bone regeneration is being researched (7). Nguyen et al. cross-linked PVA/gelatin with genipin in rabbit femurs, increasing porosity to 92% ± 2.46%. Bone formation began 5-15 days post-implantation, with a 32.67% higher bone formation than controls. The compressive strength of PVA/gelatin was significantly higher at 7.54 MPa. Increased bone strength with PVA-genipin is due to intermolecular hydrogen bonds (8). PVA also showed higher bending and compression strength compared to HAP without a cross-linker. Multivariate analysis revealed that 0.5% PVA had significant bending strength, likely due to lower toxicity and high affinity for host tissue. PVA/chitosan with HAP increased compressive modulus (27). Kamarul et al. found no inflammatory response with PVA and chitosan in rats, although histology showed acute inflammation, crucial for bone formation (9). Nguyen et al. noted reduced swelling and no inflammation after 5 and 15 weeks (8).

Limitations and Suggestion for Future Study

This study faced several limitations. Conducting research in vitro may not fully represent in vivo conditions where biological interactions could affect results. The focus on specific cross-linker concentrations limited exploration

of broader ranges or alternative agents that might improve biomechanical properties or reduce toxicity. Additionally, a limited sample size may influence the statistical robustness of the findings.

Future studies should incorporate in vivo models to better understand physiological interactions and long-term effects of cross-linked BHA with secretomes. Expanding the concentration range of cross-linkers and exploring alternative biocompatible agents could uncover more effective approaches to enhancing biomechanical stability and reducing toxicity. Increasing the sample size in future studies would improve the statistical power and reliability of the results, potentially leading to stronger and more applicable conclusions for clinical use. These steps could provide a clearer understanding of BHA's potential in orthopedic and regenerative medicine applications.

CONCLUSION

The biomechanical properties of HAP are enhanced by 0.5% PVA, which improves both compressive and bending strengths. GTA's effectiveness varies with its amount; however, higher levels may lead to toxicity. Achieving an optimal amount is essential for safe clinical application.

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