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ORIGINAL ARTICLE

Effects of Different Crosslinkers on the Release Levels of Growth Factors (TGF-ß, BMP and PDGF) From Hydroxyapatite Combined With Secretomes

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Abstract

Background/Aim: Controlled release of growth factors is essential for bone regeneration, requiring biomaterials with high stability and osteoconductivity. Hydroxyapatite (HA) is biocompatible and osteoconductive but limited by weak mechanical strength and uncontrolled growth factor release. This study evaluated the release profiles of transforming growth factor-beta (TGF-ß), bone morphogenetic protein (BMP) and platelet-derived growth factor (PDGF) from HA combined with secretome and crosslinked using polyvinyl alcohol (PVA), glutaraldehyde (GA), or aluminium hydroxide gel (Alhydrogel), aiming to identify the most effective crosslinker for controlled release and scaffold stability.

Methods: *In vitro* tests were conducted on four groups (n = 6 each): HA + PVA 0.5 % + Secretome, HA + GA 0.1 % + Secretome, HA + Alhydrogel 0.1 % + Secretome and a non-crosslinked control. HA derived from bovine bone was sterilised using gamma radiation, then crosslinked through immersion and dry-heat treatment. Samples were incubated in PBS at 37 °C and growth factor release was analysed using ELISA on days 1, 3, 7, 10 and 14.

Results: HA + PVA showed the highest TGF-ß release (617.90 ± 18.66 ng/L) and the most stable BMP (0.19 ng/L) and PDGF (0.61 ng/L) profiles. HA + Alhydrogel had the highest BMP release (6.01 ± 0.50 ng/L) and stable TGF-ß (7.57 ng/L), while HA + GA had the highest PDGF release (27.27 ± 1.92 ng/L). The control showed rapid but unstable release.

Conclusion: HA combined with secretome and PVA offers the most stable and sustained release, supporting its use as an optimal scaffold for bone regeneration.

Key words: Durapatite; Secretome; Crosslinker; Sustained release; Intercellular signalling peptides and proteins; Bone regeneration.

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Introduction

Bone tissue regeneration is a complex process requiring biomaterials exhibiting three essential properties: osteogenic, osteoconductive and osteoinductive. Unfortunately, most available bone grafts, whether natural or synthetic, do not possess all three properties simultaneously. To effectively support the bone healing process, it is crucial to develop biomaterials that can efficiently replace damaged bone tissue by fulfilling these requirements. Hydroxyapatite (HA) is a widely utilised biomaterial in bone grafting techniques, available in forms such as autografts, xenografts

and synthetic variants. HA has advantages in its biocompatibility but also has weaknesses, such as a lack of osteoinductive properties. Autografts are known as the best option due to their osteogenic, osteoconductive and osteoinductive nature, but their use is limited by the availability of tissue. Allografts face supply challenges and xenografts require processes to reduce their immunogenicity. Bovine-derived HA has potential as a biomaterial, but it naturally lacks osteoconductive properties, making it less than optimal for bone regeneration.[1, 2]

Secretomes—a mixture of bioactive molecules derived from stem cells—have been incorporated into HA to enhance its bioactivity. Recent studies highlight that secretome from mesenchymal stem cells provides a promising cell-free strategy for bone tissue regeneration, as it contains bioactive growth factors and extracellular vesicles capable of stimulating osteogenesis and angiogenesis.[3, 4] The secretome derived from adipose-derived stem cells (ADSCs) contains a diverse array of growth factors, including bone morphogenetic protein (BMP), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). Utilising secretome offers the advantage of a multi-growth factor composition, providing a more complex and comprehensive biological stimulation for bone regeneration. BMP is crucial for stimulating osteoblast differentiation, PDGF promotes cell proliferation and angiogenesis, while TGF-β plays a vital role in bone tissue remodelling. The combination of secretome and crosslinkers is anticipated to yield an effective biomaterial that optimally supports bone regeneration.[5-7]

Crosslinkers in hydroxyapatite form covalent bonds connecting functional groups of one polymer chain to another through molecular interactions, thereby enhancing biomechanical strength and intermolecular bonding. Glutaraldehyde (GA), polyvinyl alcohol (PVA) and aluminium hydroxide gel (Alhydrogel) are commonly used crosslinkers due to their biocompatible, reactive and efficient properties. These materials can form strong covalent bonds with amino or hydroxyl groups on biomaterials, such as proteins or polymers, thus improving mechanical strength, chemical stability and resistance to degradation.[8, 9]

This study investigated the effects of different crosslinkers on the release profiles of TGF-β, BMP and PDGF from hydroxyapatite combined with secretomes. The hypothesis is that different crosslinkers will affect the release levels of these growth factors, with the ultimate goal of optimising biomaterial performance for clinical applications.

Methods

Sample and design study

This study was an *in vitro* experimental study designed to evaluate the release of growth factors from HA crosslinked using three different types of crosslinkers. The HA combinations with secretome from MSCs and crosslinked were: poly-vinyl alcohol (0.5 %); glutaraldehyde (0.1 %); aluminium hydroxide gel (0.1 %); along with a control group (no crosslinking). The study sample size was determined using the Federer formula, resulting in a total of 24 samples (6 replications per group). The research was conducted at the Tissue Bank Laboratory, RSUD Dr Soetomo, the Research Centre for Vaccine Technology and Development (RCVTD) at Universitas Airlangga and the Department of Materials and Metallurgical Engineering at Institut Teknologi Sepuluh from October 2024 to January 2025.

Bovine HA preparation

Bovine HA was obtained from Bank Jaringan RSUD Dr Soetomo, where the bone was selected, cut into the desired shape and thoroughly washed to remove impurities. The bone was then subjected to a heating process at 1000 °C to ensure sterilisation and maintain its structural integrity. Following this, the HA underwent freeze-drying, beginning with deep freezing at -80 °C for 24 h to preserve its structure, followed by lyophilisation at -100 °C for 48 h to remove moisture and enhance porosity. After completing the freeze-drying process, the HA was sterilised using gamma radiation (15 kGy) at PTBGN-BATAN (Pusat Teknologi Bahan Galian Nuklir – Badan Tenaga Nuklir Nasional), Jakarta, Indonesia ensuring that the final material was free from contaminants and suitable for biomedical applications.

Crosslinking procedure

The sterilised HA was divided into three experimental groups and subjected to crosslinking using different agents: PVA 0.5 %, GA 0.1 % and Alhydrogel 0.1 %. Each HA sample was fully immersed in its respective crosslinker solution un-

der sterile conditions to allow the crosslinking agent to interact thoroughly with the HA matrix. The samples were left in the solution for 24 h to ensure optimal bonding. After the soaking process, the crosslinked HA samples underwent dry-heat treatment (DHT) at 120 °C for 30 min to facilitate covalent bond formation between the HA and the crosslinker. This step aimed to enhance the mechanical properties, stability and biocompatibility of the HA, making it more suitable for bone tissue engineering applications.

ELISA procedure

To evaluate the release of growth factors, the crosslinked HA samples were immersed in PBS and placed in a magnetic stirrer set at 37 °C to simulate physiological conditions. The release profile of TGF-β, BMP and PDGF was assessed over a 14-day period. At specific time points (days 1, 3, 7, 10 and 14), 1 cc of supernatant was collected from each sample and stored under appropriate conditions before analysis. The collected samples were analysed using an ELISA reader, which measured optical density (OD) values corresponding to the concentration of each growth factor. These OD values were then converted into ng/L concentrations using a standard calibration curve. The results provided insights into the release dynamics of growth factors, helping to determine which crosslinking method yielded the most stable and sustained growth factor release. This study aimed to support the potential clinical application of crosslinked HA as a biomaterial for bone regeneration and tissue engineering.

Statistical analysis

Quantitative data were analysed using IBM SPSS Statistics version 26. Data normality and homogeneity were assessed using Shapiro–Wilk and Levene's tests. One-way ANOVA was performed to evaluate differences among groups. A p-value < 0.05 was considered statistically significant.

Results

TGF-β

The release of TGF-β from the various treatment groups was evaluated over the 14-day period. The ELISA analysis in Table 1 showed that the HA + PVA 0.5 % + Secretome group had the highest average TGF-β release at 617.90 ± 18.66 ng/L, indicating optimal support for bone tissue regeneration. PVA effectively maintained a consistent release compared to other groups. The release profile showed a gradual increase in TGF-β levels during the initial days, peaking at day 3, followed by a slight decline and stabilisation towards the end of the observation period. Regarding stability, the HA + Alhydrogel 0.1 % + Secretome group exhibited the smallest standard deviation (7.57 ng/L), indicating more stable release over time. Conversely, the HA + GA 0.1 % + Secretome group had a lower average release of TGF-β at 546.92 ± 17.85 ng/L, suggesting that GA may not be as effective in sustaining TGF-β release compared to PVA. The control group (HA + Secretome) showed an average release of 572.17 ± 32.13 ng/L but with significant fluctuations, indicating an uncontrolled release pattern. Statistical analysis indicated no significant differences among groups ($p > 0.05$), but trends shown in Figure 1 suggest that PVA is the best crosslinker for stable TGF-β release, followed by Alhydrogel.

Table 1: Transforming growth factor-beta (TGF-β) levels from ELISA test results

N	*Crosslink* combination	Evaluation time					Mean ± SD	p-value*
		D1	D3	D7	D10	D14		
1	HA + PVA 0.5 % + Secretome	614.35	642.86	614.35	626.93	585.93	617.90 ± 18.66	0.553
2	HA + GA 0.1 % + Secretome	542.38	535.07	542.38	549.19	579.74	546.92 ± 17.85	0.903
3	HA + Alhydrogel 0.1 % + Secretome	588.26	574.92	588.26	583.43	597.69	585.20 ± 7.57	0.943
4	HA + Secretome	605.81	569.97	605.81	578.36	593.86	572.17 ± 32.13	0.436
	P-value**	0.215	0.336	0.215	0.293	0.943		

** Statistical comparison of TGF-β levels across all time points within each crosslink combination; ** Statistical comparison of TGF-β levels among all crosslink combinations at each specific time point (D1, D3, D7, D10, D14); D1: day 1; SD: standard deviation; HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel;*

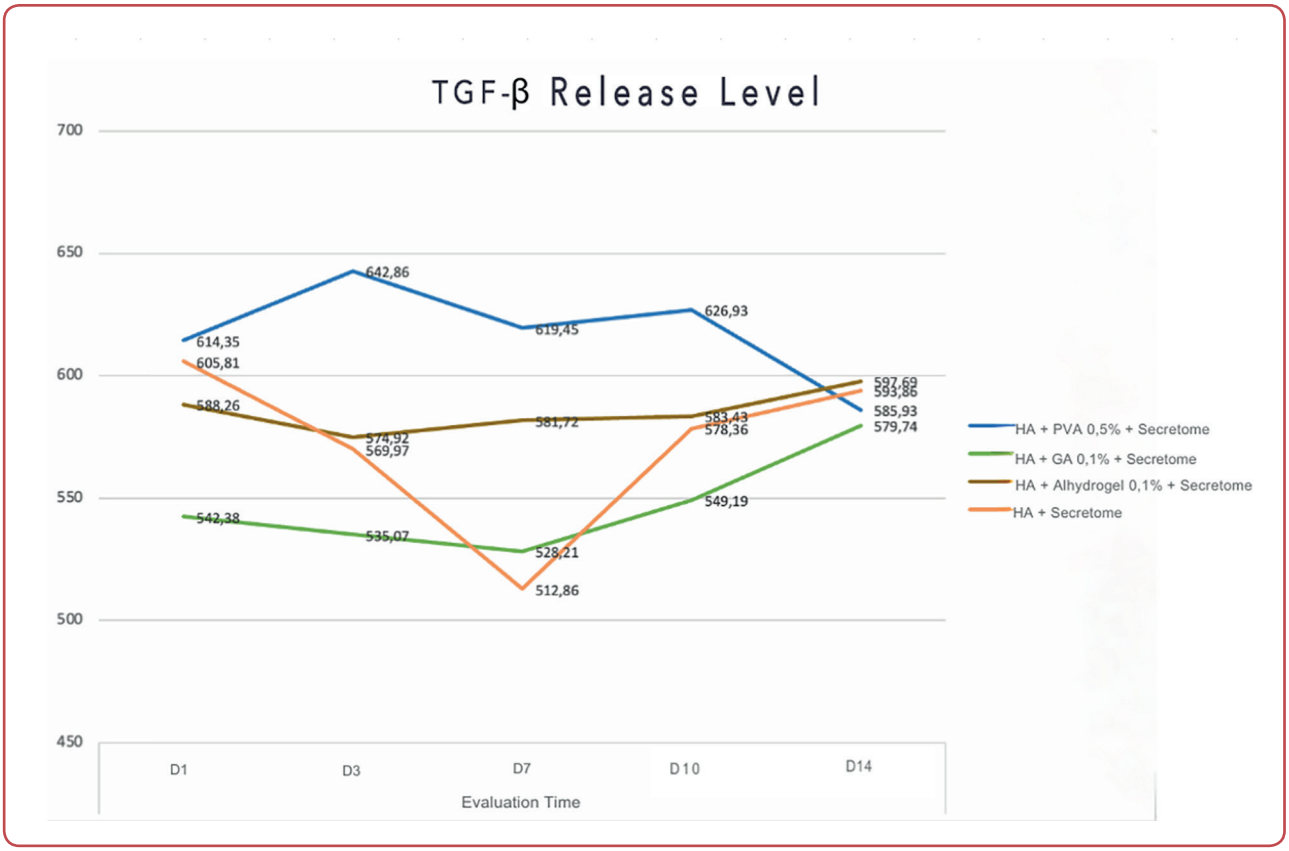

Figure 1: Evaluation of transforming growth factor-beta (TGF-β) release levels in the HA + Crosslink + Secretome and control groups HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel; D1: day 1;

BMP

The ELISA results for BMP levels, as shown in Table 2, reveal variations in release among treatment groups from days 1 to 14. The HA + Alhydrogel 0.1 % + Secretome group had the highest average release at 6.01 ± 0.50 ng/L. This suggests that Alhydrogel is particularly effective in facilitating BMP release, which is crucial for osteogenesis. The release profile indicated a steady increase in BMP levels, with the highest concentration observed at day 10. However, the HA + PVA 0.5 % + Secretome group demonstrated the smallest standard deviation at 0.19 ng/L, reflecting a more stable release and confirming PVA's effectiveness as a crosslinker.

The HA + GA 0.1 % + Secretome group showed an average release of 5.93 ± 0.44 ng/L, slightly lower than Alhydrogel but more stable than the control group, which had the lowest average release at 5.46 ± 0.35 ng/L and higher variability. Statistical analysis confirmed normal distribution and homogeneity across groups, with One-way ANOVA showing no significant differences in BMP levels

Table 2: Bone morphogenetic proteins (BMP) levels from ELISA test results

N	*Crosslink* combination	Evaluation time					Mean ± SD	p-value*
		D1	D3	D7	D10	D14		
1	HA + PVA 0.5 % + Secretome	5.68	5.92	6.07	5.88	6.24	5.96 ± 0.19	0.860
2	HA + GA 0.1 % + Secretome	5.51	5.42	6.45	6.42	5.87	5.93 ± 0.44	0.404
3	HA + Alhydrogel 0.1 % + Secretome	5.99	6.81	5.24	6.13	5.86	6.01 ± 0.50	0.064
4	HA + Secretome	5.17	5.98	5.68	5.00	5.46	5.46 ± 0.35	0.501
	P-value**	0.370	0.279	0.135	0.248	0.527		

** Statistical comparison of BMP levels across all time points within each crosslink combination; ** Statistical comparison of BMP levels among all crosslink combinations at each specific time point (D1, D3, D7, D10, D14); D1: day 1; SD: standard deviation; HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel;*

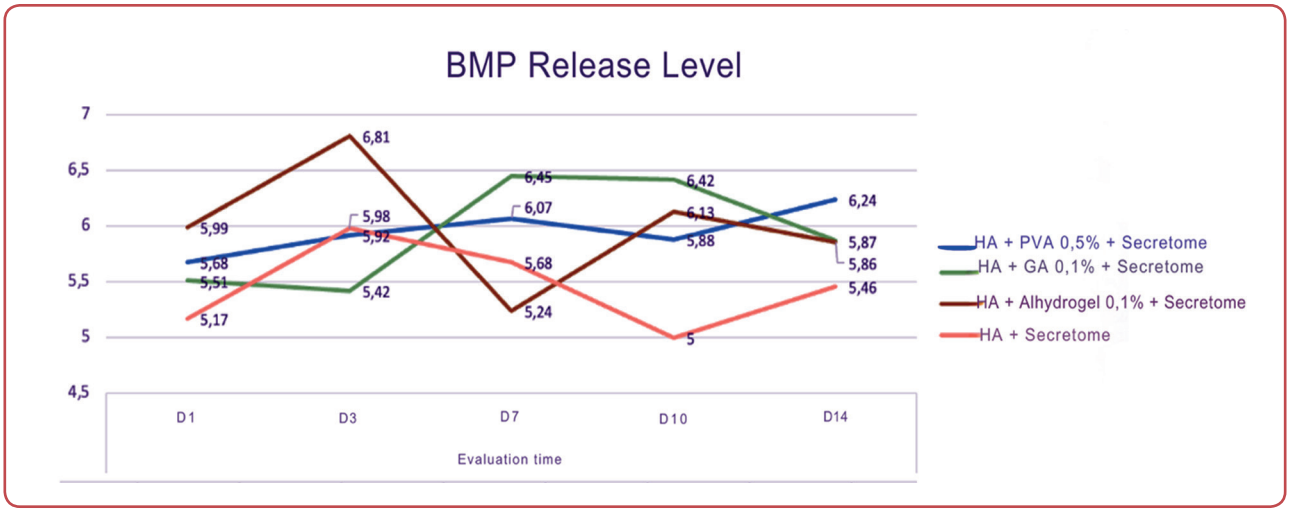

Figure 2: Evaluation of bone morphogenetic proteins (BMP) release levels in the HA + Crosslink + Secretome and control groups HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel; D1: day 1;

($p > 0.05$). Overall, Alhydrogel provided the highest average release, while PVA offered the best stability, making both crosslinkers preferable for controlled BMP release, essential for effective bone regeneration. Figure 2 illustrates the trends of BMP release levels in the HA + Crosslink + Secretome and control groups.

PDGF

The release of PDGF varied among treatment groups from days 1 to 14. The HA + GA 0.1 % + Secretome group had the highest average release at 27.27 ± 1.92 ng/L, indicating that GA supports higher PDGF release, based on Table 3. However, the HA + PVA 0.5 % + Secretome group showed the smallest standard deviation at 0.61 ng/L, reflecting a more consistent release. The HA + Alhydrogel 0.1 % + Secretome group had an average release of 26.95 ± 1.71 ng/L, slightly lower than the GA group but with better stability. While, the control group (HA + Secretome) had the lowest at 26.51 ± 2.65 ng/L, with the highest variability. Statistical analysis confirmed normal distribution, with One-way ANOVA showing significant differences at D14 ($p = 0.014$). Overall, GA 0.1 % provided the highest release, while PVA 0.5 % offered the best stability, underscoring the role of crosslinkers in regulating PDGF release for bone regeneration, the trends shown in Figure 3.

Overall, the results indicate that while PVA provides the most consistent release across all growth factors, GA is effective in achieving the highest release levels, particularly for PDGF. The Alhydrogel group also shows promise, especially for BMP release.

Table 3: Platelet-derived growth factor (PDGF) levels from ELISA test results

N	*Crosslink* combination	Evaluation time					Mean ± SD	p-value*
		D1	D3	D7	D10	D14		
1	HA + PVA 0.5 % + Secretome	26.63	25.09	26.45	26.45	25.54	26.03 ± 0.61	0.929
2	HA + GA 0.1 % + Secretome	24.16	29.18	27.16	26.46	29.38	27.27 ± 1.92	0.325
3	HA + Alhydrogel 0.1 % + Secretome	27.85	25.32	29.82	26.49	25.29	26.95 ± 1.71	0.027
4	HA + Secretome	29.08	23.70	27.96	28.88	22.93	26.51 ± 2.65	0.071
	P-value**	0.212	0.325	0.566	0.420	0.014		

** Statistical comparison of PDGF levels across all time points within each crosslink combination; ** Statistical comparison of PDGF levels among all crosslink combinations at each specific time point (D1, D3, D7, D10, D14); D1: day 1; SD: standard deviation; HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel;*

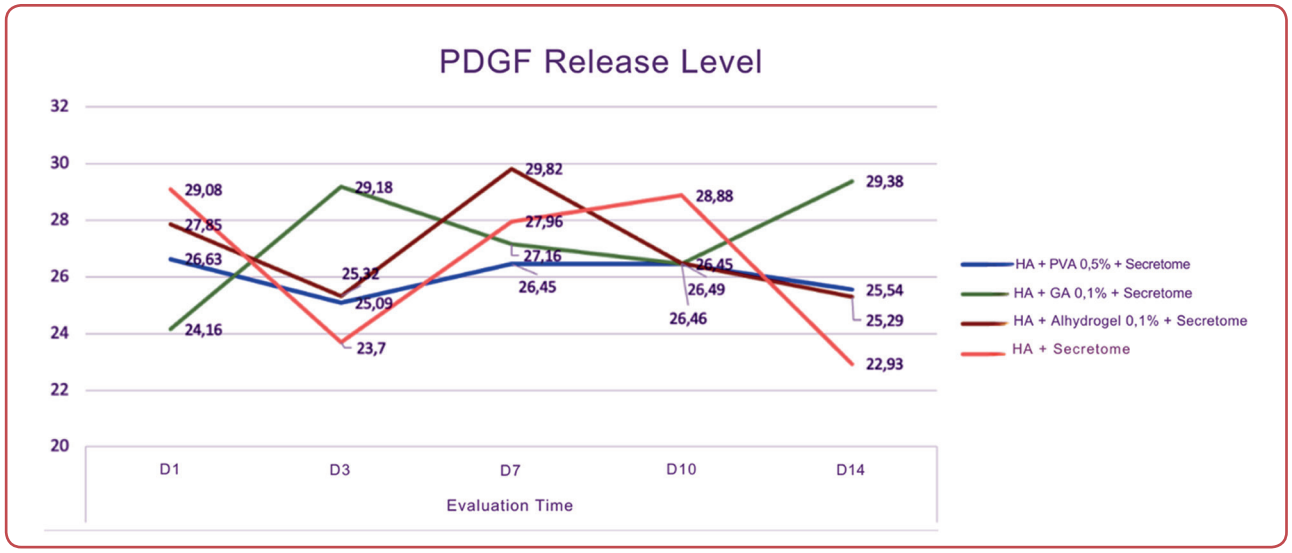

Figure 3: Evaluation of platelet-derived growth factor (PDGF) release levels in the HA + Crosslink + Secretome and control groups HA: hydroxyapatite; PVA: polyvinyl alcohol, GA: glutaraldehyde; Alhydrogel: aluminium hydroxide gel; D1: day 1;

Discussion

HA and secretome have significant potential in slow-release systems for regenerative therapy applications. HA's natural porous structure allows it to act as a carrier for bioactive molecules, ensuring controlled retention and release. These findings are consistent with recent work emphasising that hydroxyapatite-based scaffolds can serve as an efficient matrix for controlled delivery of growth factors, optimising the regeneration microenvironment.[10] Its osteoconductive and biocompatible properties facilitate the gradual diffusion of secretome, while small pores and ionic interactions help prolong the release duration.[1] The secretome, which contains growth factors and cytokines, requires a controlled release system to maintain bioactivity and enhance stability. Combining HA with polymers like PVA optimises slow release through matrix degradation and diffusion mechanisms, ensuring long-term benefits in tissue regeneration.[9]

This study evaluated the effectiveness of different crosslinkers (PVA, GA and Alhydrogel) in supporting the sustained release of TGF-β, BMP and PDGF from HA + Secretome. The results showed that PVA provides the most stable and consistent release, followed by Alhydrogel, while GA resulted in more fluctuating release patterns. The non-crosslinked control group exhibited uncontrolled release, making it unsuitable for bone regeneration applications. Several studies have confirmed that secretomes from mesenchymal stem cells (MSCs) contain bioactive factors that contribute to tissue regeneration.[5, 6] Recent molecular studies have detailed how bioactive peptides and growth factors—particularly TGF-β and PDGF—modulate osteogenic differentiation and vascular remodeling.[11]

Angiogenesis plays a crucial role in early bone regeneration and secretomes from adipose-derived MSCs have shown greater tubulogenic efficiency than those from bone marrow MSCs, primarily due to the presence of angiogenic factors such as IGF-1, VEGF-D and IL-8. These factors enhance osteogenesis and promote new bone formation, particularly in large bone defects.[12] Moreover, the presence of microvessels is known to accelerate osteoclast-mediated remodelling and increase osteoblast activity, further promoting the transition from woven bone to mature lamellar bone.[13]

Presented findings indicate that each crosslinker affects growth factor release differently. The HA + PVA 0.5 % + Secretome group showed the highest average TGF-β release (617.90 ± 18.66 ng/L), while HA + Alhydrogel 0.1 % + Secretome demonstrated the most stable release (7.57 ng/L). For BMP, HA + Alhydrogel 0.1 % + Secretome had the highest average release (6.01 ± 0.50 ng/L), but HA + PVA 0.5 % + Secretome showed the most stable release (0.19 ng/L). In terms of PDGF, HA + GA 0.1 % + Se-

cretome resulted in the highest average release (27.27 ± 1.92 ng/L), whereas HA + PVA 0.5 % + Secretome demonstrated the most stable profile (0.61 ng/L).

These findings align with previous research on HA-based scaffolds. Similarly, recent experimental studies using bovine hydroxyapatite combined with secretome demonstrated favourable release profiles of FGF-2, IL-6 and IL-10, supporting the synergistic effects of HA-secretome composites.[14] Studies on mesenchymal stem cell (MSC)-derived secretome have demonstrated its role in promoting new bone formation by stimulating angiogenesis and osteoblast activity. Hsiao et al reported that adipose-derived MSC secretome enhances tubulogenesis more effectively than bone marrow-derived MSC secretome, attributed to higher expression of IGF-1, VEGF-D and IL-8. Additionally, research on PVA-crosslinked HA scaffolds has highlighted their ability to enhance mechanical stability and regulate biomolecule release. Meanwhile, concerns over GA's cytotoxicity and inconsistent degradation have been well-documented, further supporting the preference for PVA in sustained release applications.[12]

Overall, HA + PVA 0.5 % + Secretome exhibited the most consistent and sustained release across all tested growth factors, making it the best candidate for controlled delivery in bone tissue engineering. However, if higher growth factor concentrations are required for specific applications, Alhydrogel and GA may serve as alternatives depending on the targeted factor. The toxicity of hydroxyapatite (HA), both with and without crosslinking, was evaluated using the MTT assay. Based on previous research by Abidharma et al, the results confirmed that HA exhibited biocompatibility within acceptable limits.[15] Statistical analysis showed that the p-values for most growth factors were > 0.05, indicating no significant differences between groups, except for PDGF at D14 ($p = 0.014$). Despite this, the observed trends suggest that PVA provides the most consistent and prolonged release, making it a preferable choice for controlled biomaterial applications.[16, 17] The importance of scaffold architecture and crosslinking conditions in achieving controlled release has also been emphasised in recent reviews on stem cell–based bone tissue engineering.[18]

These results support the potential of crosslinked HA + Secretome with PVA in bone regeneration by enabling sustained growth factor release, essential for different phases of bone healing from inflammation to remodelling. PVA is the most promising crosslinker for clinical applications in bone tissue engineering, followed by Alhydrogel, while GA requires further optimisation to improve release consistency.[19, 20]

However, this study has limitations. As an in vitro experiment, it does not fully replicate the complex physiological environment of bone healing. Further in vivo studies are necessary to assess biocompatibility, biodegradability and the long-term effects of crosslinked HA + Secretome in bone defect models. Additionally, longer observation periods are needed to evaluate sustained release patterns and hybrid crosslinking approaches (eg PVA + Alhydrogel) should be explored to optimise release kinetics for improved clinical outcomes.

Conclusion

This study found that HA + PVA 0.5 % + Secretome provided a more stable release of TGF-β, BMP and PDGF, whereas GA and Alhydrogel led to faster but less stable release. PVA proved to be the best crosslinker for maintaining gradual growth factor release, making it a strong candidate for bone tissue engineering applications. No significant differences were observed in TGF-β and BMP release across all groups at various time points, but PDGF release at day 14 was significantly higher in crosslinked groups compared to non-crosslinked HA + Secretome.

For future research, *in vivo* studies are recommended to assess clinical applicability, along with extended observation periods for long-term growth factor release. Further optimisation of biocompatibility, stability and safety of HA + Secretome crosslinked with PVA is needed, as well as exploration of hybrid crosslinking strategies (eg PVA + Alhydrogel) to enhance controlled release.

Ethics

This study received ethical clearance from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga. The ethical clearance certificate was issued under the number 2.KEH.2.01.2025, dated 15 January 2025, confirming the study's compliance with ethical standards.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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