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Original Article

Growth Factor Comparison in Cortical Demineralized Bone Matrix that Demineralized Using Chloric and Acetic Acid

Mouli Edward[1,2,3], Ferdiansyah Mahyudin[1,2,3], Mohammad Hardian Basuki[1,2,3], Heri Suroto[1,2,3], Ferdiansyah Danang Perwira[1,2]

[1]Department of Orthopedics and Traumatology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[2]Department of Orthopedics and Traumatology, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia
[3]Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia

Correspondence should be addressed to Mouli Edward, Department of Orthopedics and Traumatology, Faculty of Medicine, Universitas Airlangga, Jl. Mayjend Prof. Dr. Moestopo No. 6-8 Surabaya 60286, Indonesia. e-mail: mouli-edward@fk.unair.ac.id

ABSTRACT

Background: Demineralized bone matrix (DBM) is an alternative biomaterial for which specific acid and immersion time are needed to optimize growth factor preservation. The optimal demineralization protocol for preserving growth factors in DBM remains unclear. This study investigated DBM extraction methods using different acids and immersion times to maintain optimal growth factor preservation.
Methods: This in vitro experimental laboratory study used a randomized controlled post-test-only group design. We characterized the Insulin-like growth factor-1 (IGF-1), Bone morphogenetic protein-2 (BMP-2), and Transforming growth factor-β (TGF-β) content of 1 gram of New Zealand White Rabbit cortical bone immersed in 0.6 M hydrochloric acid and 0.5 M acetic acid for 3, 6, and 9 days. We then analyzed the differences in growth factor levels in each acid and performed statistical analysis.
Results: IGF-1 levels were higher in DBM demineralized with acetic acid than with hydrochloric acid. BMP-2 and TGF-β levels were higher in DBM demineralized using hydrochloric acid. The concentration of growth factors decreased over time in DBM demineralized using acetic acid. The highest growth factor level was obtained after 6 days of immersion in hydrochloric acid.
Conclusion: DBM demineralized with acetic acid yielded higher average IGF-1 levels compared to hydrochloric acid. However, BMP-2 and TGF-β levels were higher with hydrochloric acid. Growth factor levels in hydrochloric acid peaked at 6 days and then decreased. These results suggest that avoiding over-demineralization is important for maintaining growth factor levels. Further research is needed to optimize DBM processing.

Keywords: Bone morphogenetic protein-2; Demineralized bone matrix; Human and medicine; Insulin-like growth factor-1; Transforming growth factor-β

INTRODUCTION

The field of regenerative medicine has generated interest in prospective bone healing procedures. Bone defects are common and can be caused by surgery, infection, congenital anomalies, and trauma. Although bones have a high capacity for self-healing, some defects or fractures are too large to heal spontaneously. Bone growth must be stimulated by various bioactive implantable materials, cellular components, and intracellular and extracellular molecular signaling pathways to rebuild the bone structures as part of patient treatment.[1] An ideal biomaterial for bone substitution, such as a bone graft, must have the following properties to improve bone healing: osteoinductive, osteoconductive, biomechanically stable, disease-free, and minimally antigenic. Bone grafts can be divided into three groups based on the preparation procedure: fresh-frozen, freeze-dried, and DBM. Fresh-frozen allografts provide the highest mechanical stability but also carry the highest risk of disease transmission.

Demineralized bone matrix (DBM) is a

biomaterial with osteoconductive and osteoinductive scaffolds that improve the healing process of various bone defects. DBM is extracted using specific acids to remove the bone mineral content, leaving non-collagenous proteins, growth factors, and type 1 collagen.[2] Many grafts reduce surgical wound complications associated with autograft harvesting. Additionally, using DBM can speed up surgery and patient recovery time.[3,4] DBM with bone marrow aspiration improves fracture recovery rates and reduces morbidity and treatment length in non-union cases.[5] DDBM has no structural component and cannot fill significant bone defects. For daily application, the most common form of DBM is paste; it can be easily shaped and is not readily dissolved in liquids. The successful application of DBM requires a well-vascularized network environment to integrate the recruitment and differentiation of stem cells.[3]

Marshall Urist first introduced DBM in the 1970s using hydrochloric acid (HCl). Bone is dissolved in acid to remove the bone matrix's mineral content. An increase in acid concentration disproportionately increases the demineralization rate. The reaction temperature, the volume of acid solvent compared to bone weight, the rate of acid dissociation, the frequency of acid replacement, volume, the thickness of demineralized bone tissue, the concentration of hydroxyapatite in bone, bone surface area, bone particle size, and degree of bone hardness also affect the demineralization rate. Knowing this, we can adjust the demineralization rate by changing these factors.[6]

The preservation of growth factors plays an essential role in DBM processing. Insulin-like growth factor (IGF), Bone morphogenetic protein (BMPs), and Transforming growth factor-β (TGF-β) play important roles in bone healing. IGF is a growth factor found in osteoblasts during intramembranous ossification and in pre-hypertrophic chondrocytes, which increases collagen synthesis (primarily type 1 collagen) and stimulates the replication of proteoblastic cells. IGF is critical in skeletal development and maintaining skeletal structure, as it is produced locally during callus formation and the repair of bone defects. IGF-1 plays an important role during fetal organogenesis and facilitates the regeneration of various tissues, such as bone, muscle, and nerves. The ability of IGF-1 to promote tissue regeneration is associated with reducing pro-inflammatory cytokines and stimulating anti-inflammatory cytokines.[7,8]

BMPs (BMP-2 and BMP-7) have been used clinically to treat fractures, non-unions, and spinal fusion. The expression of BMP-2 and BMP-4 increases, and osteoblasts begin to lay down the osteoid parallel to the tension line vector, initiating bone regeneration.[9] BMP is a growth factor that induces primitive mesenchymal and osteoprogenitor cells. BMPs are abundant in undifferentiated mesenchymal cells during the inflammatory phase and are also found in osteoblasts during intramembranous ossification. BMP-2 and BMP-4 help to activate osteoblasts and start osteoid formation as the initial process of bone regeneration.[10]

TGF-β positively affects fracture healing. The systemic and local injection of TGF-β can increase callus size and strength. Lind et al. found that TGF-β increased callus strength and size in rabbits with tibial defects, whereas TGF-β did not affect stiffness, bone mineral content, and Haversian canal diameter. Overall, the stimulatory effect of TGF-β on fracture healing appears small and may be related to a member of its protein superfamily BMP.[11] TGF-1 and TGF-2 were identified as cartilage-promoting factors in bone extracts in the muscle cell chondrogenesis assay.

BMP and TGF-β can affect osteogenesis through direct and indirect bone healing, mainly by activating osteoblasts.[12,13] BMP-2 also increases the IGF-1 expression from osteoblasts. BMP levels in DBM demineralized with hydrochloric acid peaked in the first 90 min and then decreased, with the osteoinductive potential of

DBM having a half-life of seven days. TGF-β is the most potent chemoattractant for macrophages produced by osteoblasts. It stimulates the differentiation of periosteal mesenchymal cells and regulates the cartilage matrix and osteoblast activity. All three are growth factors crucial in bone healing and can provide clues about the osteoconductive potential of DBM.

Hydrochloric acid (HCl) and ethylene diamine tetra acetic acid (EDTA) often process bone into DBM. Other acids that can be used to demineralize bones are formic and acetic. Acetic acid has good potency and has yet to be studied extensively. Acetic acid reacts with calcium phosphate to produce calcium acetate and phosphoric acid.[9]

The process of bone demineralization requires a balance between the acid type and optimal soaking time to preserve the growth factors. Although DBM has been used clinically for the last 15 years, the characteristics of DBM demineralized using different acids has been rarely discussed. In this study, we aimed to compare the osteoconductive and osteoinductive features of the demineralized bone matrix with hydrochloric and acetic acids by assessing the growth factors contained therein. According to previous studies, the osteoinductive potential of DBM has a half-life of 7 days when immersed in hydrochloric acid. Therefore, we will make observations on days 3, 6, and 9 after demineralization.

MATERIAL AND METHODS

An in vitro experimental laboratory study was performed to compare the growth factors of cortical bone immersed in hydrochloric acid and acetic acid. A randomized controlled post-test-only group design was utilized. The experimental unit consisted of three groups, each comprising eight 1 cm^3 samples of fresh-frozen cortical bone from a New Zealand white rabbit. It was assumed that each sample was homogenous and exhibited the same baseline characteristics.

Bone demineralization

All soft tissue attached to the bone was dissected, and the bone was cut to the designated size (1 cm^3). The first washing was performed using distilled water by jet lavage to remove all blood and bone marrow. In the second wash, the bone was submerged in hydrogen peroxide (H_2O_2), placed on a water bath ultrasonic shaker for 24 h, and heated to 70°C.

The bone underwent a third washing process with distilled water and was then submerged in a hexane solution to remove the remaining fat. The mixture was rewashed until the hexane solution was dissolved and removed. The demineralization process was initiated by immersing the cleaned bones in hydrochloric and acetic acid, where 0.2 cc of each acid was dissolved in 0.9% Sodium Chloride (NaCl) to a final volume of 1000 cc, and the pH of the solution was confirmed to be below 4. Each sample was soaked in 10 cc of the solution until complete demineralization occurred. The soaking solution was changed daily to maintain a pH of less than 4.

The fourth washing process was performed to remove any of the residual hydrochloric acid and acetic acid solutions using distilled water. The demineralized bone matrix (DBM) was then frozen at -80°C for 24 and 72 h. Afterwards, it was placed in a dryer for 48-50 hours until the water content level was below 8%.

Growth Factor Quantity Analysis

The quantity of growth factors formed after demineralization was measured using an enzyme-linked immunosorbent assay (ELISA) specific to each growth factor. Detection was performed by measuring the activity of the conjugated enzymes through incubation with the substrate to generate a measurable product. The ELISA (Bio-Rad) examination was performed at the Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

Statistical Analysis

Data distribution was determined to be normal based on the Shapiro-Wilk and Kolmogorov-Smirnov tests. The data was further analyzed using two-way repeated measures ANOVA to determine significant differences in IGF-1, BMP-2, and TGF-β levels between group 1 (cortical bone demineralized in HCl) and group 2 (cortical bone demineralized in acetic acid).

The results of the multivariate tests (between-subjects effect and within-subjects effect) were used to analyze the relationship between the growth factor levels, the type of acid used in the demineralization process, and the duration of submersion. Between-subjects analysis evaluated the growth factors between the two groups (cortical bone demineralized using acetic acid compared to cortical bone demineralized using hydrochloric acid), regardless of submersion time (3, 6, or 9 days). Four tests were performed for the between-subjects analysis: Pillai's Trace, Wilks' Lambda, Lawley-Hotelling Trace, and Roy's Largest Root. A significance value of < 0.05 (0.000) was obtained from the four tests, indicating a significant difference in growth factor values between cortical bone demineralized with hydrochloric acid compared to cortical bone demineralized using acetic acid, regardless of immersion time.

Ethical Approval

Ethical approval was granted by the Research Ethics Commission of the Faculty of Veterinary Medicine, of Airlangga University's Animal Care and Use Committee (ACUC) (No. 2.KE.064.08.2020).

RESULTS

Eight samples were used for each study parameter. The mean values for each parameter are presented in Table 1.

Table 1. Mean value of growth factors.

Growth Factors	Day	Acid	Mean (ng/ml)
IGF-1	Day 3	Hydrochloric Acid	555.94 ± 21.56
		Acetic Acid	594.79 ± 36.13
	Day 6	Hydrochloric Acid	574.27 ± 40.27
		Acetic Acid	587.25 ± 18.39
	Day 9	Hydrochloric Acid	556.10 ± 39,08
		Acetic Acid	560.65 ± 46.38
BMP-2	Day 3	Hydrochloric Acid	0.089 ± 0.0054
		Acetic Acid	0.084 ± 0.0056
	Day 6	Hydrochloric Acid	0.100 ± 0.0059
		Acetic Acid	0.082 ± 0.0057
	Day 9	Hydrochloric Acid	0.088 ± 0.0048
		Acetic Acid	0.079 ± 0.0063
TGF-β	Day 3	Hydrochloric Acid	0.666 ± 0.0363
		Acetic Acid	0.671 ± 0.0520
	Day 6	Hydrochloric Acid	0.707 ± 0.0502
		Acetic Acid	0.639 ± 0.0317
	Day 9	Hydrochloric Acid	0.631 ± 0.0439
		Acetic Acid	0.600 ± 0.0244

The average concentration of IGF-1 in hydrochloric acid immersion was 555.94 ng/ml, 574.27 ng/ml, and 556.10 ng/ml on days 3, 6, and 9, respectively. The average concentration of IGF-1 in acetic acid immersion was 594.79 ng/ml, 587.25 ng/ml, and 560.65 ng/ml on days 3, 6, and 9, respectively. The average concentrations of BMP-2 in the hydrochloric acid immersion were 0.089, 0.100, and 0.088 ng/ml on days 3, 6, and 9, respectively. The average concentrations of BMP-2 in acetic acid immersion were 0.084, 0.082, and 0.088 ng/ml on days 3, 6, and 9, respectively. The average concentrations of TGF-β in the hydrochloric acid immersion were 0.666, 0.707, and 0.631 ng/ml on days 3, 6, and 9, respectively. The average concentrations during acetic acid immersion were 0.671 ng/ml, 0.639 ng/ml, and 0.600 ng/ml on days 3, 6, and 9, respectively.

Data distribution was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests and was determined to be normal. A two-way repeated measures ANOVA was conducted to determine if significant differences in growth factor values existed between group 1 (cortical bone immersed in hydrochloric acid) and group 2 (cortical bone immersed in acetic acid).

DBM demineralized using acetic acid showed a decrease in the concentration of IGF-1 over time. The highest IGF-1 value was found on day 3, and the lowest on day 9. The value of IGF-1 in DBM demineralized using hydrochloric acid was higher on day 6 compared to days 3 and 9, with day 9 showing a greater value than day 3 (Figure 1). BMP-2 levels in DBM demineralized with hydrochloric acid were highest on day 6 compared to days 9 and 3, with day 9 showing a greater value than day 3. BMP-2 levels in DBM demineralized using acetic acid decreased with the increasing immersion time; day 6 showed the highest value compared to days 3 and 9 (Figure 2).

TGF-β levels in DBM demineralized using hydrochloric acid were highest on day 6 compared to days 9 and 3, with day 3 showing a greater value than day 9. The value of TGF-β in DBM demineralized using acetic acid decreased with an increasing immersion time, with day 6 showing the highest value compared to days 3 and 9 (Figure 3).

The analysis of growth factors in DBM demineralized on days 3, 6, and 9 within the same acid group was conducted using Pillai's Trace, Wilks' Lambda, Lawley-Hotelling Trace, and Roy's Largest Root tests. A significance value of < 0.05 (0.001) was obtained from these four tests, indicating a significant difference in growth factor values between the cortical bone demineralized for 3, 6, and 9 days within the

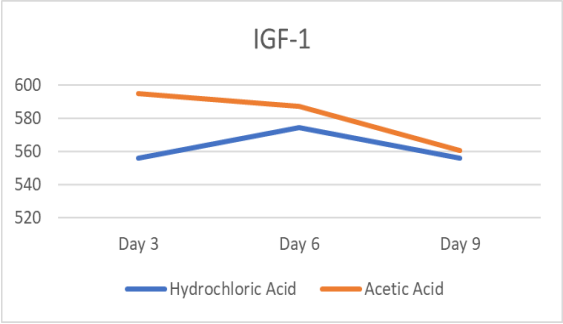

Figure 1. IGF-1 concentration level in hydrochloric acid and acetic acid on 3, 6, and 9 days immersion.

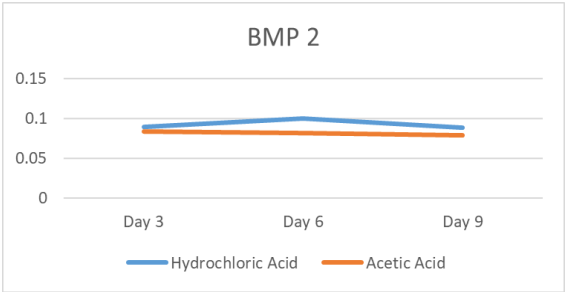

Figure 2. BMP-2 concentration level in hydrochloric acid and acetic acid on 3, 6, and 9 days immersion.

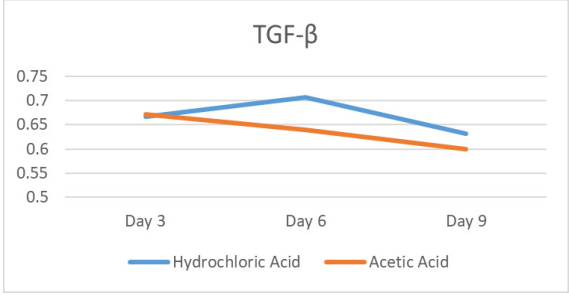

Figure 3. TGF-β concentration level in hydrochloric acid and acetic acid on 3, 6, and 9 days immersion.

Table 2. Effects of Acid Type and Demineralization on Growth Factors*.

Source of Variation	*p* value
Tests of Between-Subjects Effect	
Intercepts	< 0.001
Groups	< 0.001
Tests of Within-Subjects Effect	
Days	0.001
Days-Groups	0.040

*Data were analyzed using Pillai's trace, Wilks' Lambda, Lawley-Hotelling Trace, Roy's Largest Root

same acid group.

The results of the within-subjects analysis for the evaluation of the growth factors on days 3, 6, and 9, comparing the hydrochloric acid and acetic acid groups using Pillai's Trace, Wilks' Lambda, Lawley-Hotelling Trace, and Roy's Largest Root tests, yielded significance values of < 0.05 (p = 0.040) (Table 2). This indicates a significant difference in growth factor values between the cortical bone demineralized for 3, 6, and 9 days when comparing the hydrochloric and acetic acid groups.

Analysis of growth factors in DBM demineralized on days 3, 6, and 9 within the same acid group was conducted using Pillai's Trace, Wilks' Lambda, Lawley-Hotelling Trace, and Roy's Largest Root tests. A significance value of < 0.05 (p = 0.001) was obtained from these four tests, indicating a significant difference in growth factor values between the cortical bone demineralized for 3, 6, and 9 days within the same acid group.

DISCUSSION

A previous study reported that demineralized bone matrix could treat bone defects and show significantly higher new bone formation, bone volume, better mechanical strength, and stiffness, alongside substantially lower inflammatory cells, fibroblasts, fibrocytes, and strain.[14,15] The study parameters we used, IGF-1, BMP-2, and TGF-β, play a role in the osteoconductivity function of DBM. IGF-1 is a growth factor found in osteoblasts during intramembranous ossification and in pre-hypertrophic chondrocytes, which increases collagen synthesis (especially type 1 collagen) and stimulates the replication of proteoblastic cells. BMP is a growth factor that produces primitive mesenchymal cells and osteoprogenitor cells. BMPs are abundant in undifferentiated mesenchymal cells during the inflammatory phase and are also found in osteoblasts during intramembranous ossification. BMP-2 also increases the IGF-1 expression in osteoblasts. TGF-β is the most potent chemoattractant for macrophages produced by osteoblasts, and it stimulates the differentiation of periosteal mesenchymal cells and regulates the cartilage matrix and osteoblast activity. All three growth factors are important in the bone healing process

Our study found that IGF-1 levels in cortical DBM demineralized with acetic acid were higher than the IGF-1 levels in cortical DBM demineralized with hydrochloric acid. On the other hand, the BMP-2 and TGF-β levels were higher in cortical DBM demineralized with hydrochloric acid than in cortical DBM demineralized with acetic acid. The IGF-1 concentration decreased with the increase in immersion time. The IGF-1 level on day 3 was higher than that on day 6, and day 6 was higher than day 9.

In contrast to acetic acid, the IGF-1 levels in DBM demineralized with hydrochloric acid were higher on day 6 than on days 3 and 9, with day 9 showing a greater value than day 3. BMP-2 levels were highest on day 6 compared to days 9 and 3, with day 9 showing a higher value than day 3.

In contrast to hydrochloric acid, BMP-2 levels in DBM demineralized using acetic acid decreased with an increase in immersion time; day 3 was higher than day 6, and day 6 was higher than day 9. TGF-β levels were highest on day 6 compared to days 9 and 3, with day 3 showing a higher value than day 9. In contrast to hydro-

chloric acid, TGF-β levels in DBM demineralized using acetic acid decreased with increasing immersion time but were higher on day 6 than on day 9. This pattern affects the morphological and functional properties of DBM for bone healing. The osteoinductive and osteoconductive activities of DBM have been attributed to glycoproteins known as BMPs, particularly BMP-2 and BMP-7, and other growth factors known to be efficient in the bone healing process. These protein-based factors promote mesenchymal stem and progenitor cell migration, as well as their proliferation, and differentiation into chondroblasts and osteoblastic lineages.[16] TGF-β may stimulate chemotaxis and the mitogenesis of osteoblasts and chondroblast precursors and inhibit osteoclast formation and bone resorption.[17] While BMPs are the essential proteins for bone induction, other growth factors have also been identified in bone, including IGF-1.

Furthermore, this was emphasized in the multivariate analyses using tests examining the between-subjects and within-subjects effects. We obtained a statistically significant value of < 0.05 (0.000) from the four tests, which indicates a significant difference in growth factor values between cortical bone demineralized with hydrochloric acid compared to cortical bone demineralized using acetic acid, regardless of immersion time. From these four tests, we also obtained a statistically significant value of < 0.05 (0.001), which indicates a significant difference in the values between cortical bone demineralized for 3, 6, and 9 days within the same acid group. Comparing the hydrochloric and acetic acid groups showed a significant difference in growth factor values between cortical bone demineralized for 3, 6, and 9 days.

The coordinated action of stem cells, mainly from the periosteum which differentiates into chondrocytes and osteoblasts, first generates the soft (cartilage) callus and then the hard (bone) callus, which is necessary for fracture healing. Vascular invasion, which is present along with these stem cells, is essential for the differentiation process and may allow the osteoclasts to enter, which are required to remodel the callus into mature bone.[18] When an injury occurs, the bone healing response occurs in five stages: the hematoma phase, inflammation, the formation of a soft callus, the formation of a hard callus, and finally, bone remodeling. In cases of extensive bone damage, the bone response decreases and disrupts the spontaneous regeneration process, requiring intervention. Bone grafts are bone replacement materials that have been widely studied and used to treat bone defects. One of the bone graft methods is demineralizing the bone matrix.[19,20] These results have shown that different acid solutions and durations may optimize sample conductivity. In a previous study, BMP levels in DBM demineralized with hydrochloric acid peaked during the first 90 min. They then decreased, with the osteoinductive potential of DBM having a half-life of seven days. Because the decrease in growth factors occurred within a certain period, it can be concluded that the growth factors contained in the cortical bone also diffused into the acid used. The phase of a transient increase in growth factor levels caused by demineralization with hydrochloric acid is possibly due to the action of proteolytic enzymes in the bone matrix. This enzyme is a type of protease and consists of an organic matrix that will degrade the growth factors after an injury to the bone. Low temperature and pH protect the growth factors against this process.[21]

Although demineralization in strong acid solutions such as HCl or HNO_3 is rapid, the safety of its direct use in living tissues has yet to be established. The demineralization of bone increases the release and bioavailability of matrix-associated proteins, resulting in osteoinductive grafts. IGF, BMP, and TGF complement the available growth factors, where BMP is the main protein known to be osteoinductive.[9]

After the multivariate analysis of the demineralized cancellous bone within subjects and

within one group, this research has made significant findings following the statistical analysis. Although conducted with different BMP-7s, previous studies noted similar results regarding the peak level of complete demineralization of the bone and the time of diminished demineralization.[21] A constant concentration of growth factors in DBM cannot be guaranteed, and fluctuations may exist between different batches. Pooling the different sets during processing could solve this problem, but our biovigilance recommendation and traceability rules are unacceptable.[9] Nonetheless, knowing this phenomenon should stimulate more research into optimizing clinical bone graft demineralization processing by tissue banks.

This study has several limitations. A larger sample size could be considered in similar experimental settings because of the mixed conclusions drawn from our results. The use of 0.9% NaCl solution as a solvent could be changed because 0.9% NaCl tends to be acidic (pH = 5). Other types of acid could be included to compare the effectiveness of different solutions in the demineralization process and to produce significantly different growth factors—a more precise measurement of time would enable the determination of the exact time for demineralization to maintain the growth factor content.

This research is expected to be a reference that can be used to determine the type of acid and soaking time used, which is beneficial for DBM production to maintain its osteoconductive and osteoinductive properties as a bone graft.

CONCLUSION

Differences in growth factors (IGF-1, BMP-2, and TGF-β) were observed in DBM demineralized using acetic acid and hydrochloric acid. DBM demineralized with acetic acid yielded higher average IGF-1 levels than that demineralized with hydrochloric acid. However, the BMP-2 and TGF-β levels were higher in the DBM demineralized with hydrochloric acid. Growth factor levels in DBM demineralized with hydrochloric acid peaked on day 6 and then decreased. In contrast, the growth factors in DBM demineralized with acetic acid decreased with time. These results suggest the importance of avoiding over-demineralization to maintain growth factor levels and the osteoinductive capacity of DBM.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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