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ORIGINAL RESEARCH

COMPARISON OF TGF-B1 AND BMP-2 CONTENTS IN CANCELLOUS BOVINE BONE PROCESSED BY FREEZE-DRYING AND DECELLULARIZATION (BIOMATERIAL PROFILE STUDY)

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ABSTRACT

Background: Xenogenic scaffold is an alternative in bone tissue engineering derived from xenograft used for the reconstruction of critical size defects in the maxillofacial region. Cancellous bovine bone processed through freeze-drying results in freeze-dried bovine bone (FDBB) scaffold. FDBB continues to be developed due to the existing risk of transmission through the decellularization process, resulting in decellularized freeze-dried bovine bone (dc-FDBB) scaffold. The freeze-drying process, the addition of chemicals in the decellularization process, and sterilization with gamma-ray radiation can reduce the levels of growth factors such as TGF-β1 and BMP-2, thereby diminishing the osteoinductive potential required in bone regeneration.
Objective: This study aims to compare the levels of TGF-β1 and BMP-2 in cancellous bovine bone processed through freeze-drying and decellularization.
Method: Extraction and quantification of growth factors were carried out on Fresh Cancellous Bovine Bone, FDBB, and dc-FDBB. Comparative tests were performed using ANOVA and Kruskal Wallis tests.
Results: The mean levels of TGF-β1 and BMP-2 were highest in Fresh Cancellous Bovine Bone (16,453 pg/mL; 10,181 pg/mL), followed by FDBB (9,300 pg/mL; 7,086 pg/mL) and dc-FDBB (6,291 pg/mL; 5,672 pg/mL). There were no significant differences in the levels of TGF-β1 and BMP-2 between FDBB and dc-FDBB ($p = 0.378$; $p = 0.429$). There were significant differences in the levels of TGF-β1 and BMP-2 between Fresh Cancellous Bovine Bone and FDBB ($p = 0.037$, $p = 0.034$), as well as Fresh Cancellous Bovine Bone and dc-FDBB ($p = 0.004$, $p = 0.003$).
Conclusion: There is a reduction in the levels of TGF-β1 and BMP-2 in cancellous bovine bone processed through freeze-drying and decellularization, but there is no significant difference in the levels of TGF-β1 and BMP-2 between FDBB and dc-FDBB.

***Keywords*:** Freeze-Drying, Decellularization, Growth Factor, TGF-β1, BMP-2

1. INTRODUCTION

In the practice of oral surgery, damage to the maxillofacial bone can be caused by tumors, infections, trauma, and congenital abnormalities. Extracting teeth or removing lesions on the bone can result in resorption and atrophy of the alveolar bone, as well as create continuity defects caused by segmental resection. Bone defects with a gap of 2 cm or more that will not heal on their own or undsergo regeneration are referred to as critical size defects. One modality for addressing this defect is reconstruction with a bone graft.[1–3]

Bone graft materials can be either natural or synthetic. Autografts are regarded as the gold standard due to their exceptional capacity to promote osteogenesis, osteoinduction, and osteoconduction. Nonetheless, their application is restricted by several challenges, including donor site morbidity, limited availability and variability in shape, as well as the potential complications that can arise from the secondary surgical procedure required for graft harvesting. One alternative is the use of allografts, sourced from donors and transformed into fresh frozen, freeze-dried bone allograft (FDBA), as well as demineralized freeze-dried bone allograft (DFDBA). There are hesitations regarding the transfer of diseases, the reaction to antigens, and the requirement for thorough compatibility checks, which are all factors leading to the reluctance to use fresh frozen bone. When FDBA is

implanted in tissue, it acts as an osteoconductive material and stimulates resorption. DFDBA, on the other hand, offers both osteoconductive and osteoinductive materials. Allografts have a benefit in that their availability is widespread, and patients do not experience any complications at the donation site. However, drawbacks involve the preparation procedure, which compromises the integrity and osteogenic capabilities of the material, the potential for transmitting diseases, and the necessity for thorough matching to minimize the chances of graft rejection and disease transmission.[4]

Xenografts have been identified as a potential alternative due to their ease of availability and unlimited supply of material, but their weakness is that they elicit a strong immune response.[5] Bovine bone xenograft is the preferred option since it is functionally and structurally similar to human bone. Due to religious and ethical concerns, the majority of Indonesians prefer bovine bone xenografts.[6] Ceramics used in synthetic bone grafts include calcium phosphate, bioglass, and calcium sulfate. These materials are inert and exhibit little to no osteoinductive activity. Synthetic grafts have the advantage of being non-antigenic and having an unlimited supply, but they are unable to encourage comprehensive bone regeneration.[7,8]

Tissue engineering is a medical technology that is continuously developing, with bone tissue engineering serving as an illustration of this progress.[7] Bone tissue engineering revolves around three essential components that facilitate the process of bone regeneration, commonly known as the tissue engineering triad: a scaffold to support cell repopulation, growth factors to stimulate the development of new tissue, and osteogenic cells tasked with forming new bone structures. The scaffold must have several properties relevant to its application, including osteogenic, osteoinductive, and osteoconductive properties.[9] Further, the scaffold must be three-dimensional, biocompatible, with good mechanical properties and a chemically favorable surface for cell attachment, proliferation, and differentiation.[10] The process of bone tissue engineering using xenografts, or foreign scaffolds, involves various methods to produce deproteinized bovine bone mineral (DBBM), freeze-dried bovine bone (FDBB), and decellularized FDBB (dc-FDBB).[11,12]

DBBM is a type of xenograft that has undergone a deproteinization treatment, which eliminates all organic bone debris. DBBM has host-friendly features; even so, all growth factor content is eliminated, making it non-osteoinductive. Given its moderate breakdown rate, DBBM has a limited bone regeneration capability. Consequently, FDBB was developed to solve the shortcomings of DBBM. FDBB, like FDBA, contains both inorganic and organic components, making it osteoconductive and osteoinductive due to the presence of numerous growth factors.[12,13]

The xenograft processed by removing fat and tissue chemically, as well as through freeze-drying procedures, produces FDBB with the aim of eliminating antigenicity and pathogenicity. In a study conducted by Kamadjaja et al.[13], FDBB from cancellous bovine bone was soaked in a 3% hydrogen peroxide solution (H2O2 3%), frozen at -80°C, and dried with a lyophilizer until the water content was below 10%. FDBB retains both inorganic and organic materials such as growth factors and other cellular components. The osteoinductive properties of FDBB are attributed to the presence of growth factors such as TGF-β, IGF, FGF, and BMP.[14] The use of H2O2 has a negative impact by reducing the level of growth factors, thereby diminishing osteoinductive ability. The decrease in growth factor levels occurs with increasing immersion time in H2O2.[15]

The process of decellularization or cell cleansing is one strategy utilized to eliminate immunogenic properties in xenogeneic scaffolds, resulting in dc-FDBB.[16] Decellularization preserves the arrangement and makeup of the extracellular matrix (ECM). Decellularized bone contains collagen, non-collagen proteins, and growth factors such as BMP and TGF-β, which confer osteoinductive properties.[8] Various characteristics and protocols for decellularization are employed by research groups, yet a standardized approach has not been established. Decellularization is possible using various techniques, including physical methods, enzymes, or treatment with chemical substances like Triton X-100, Sodium Lauryl Ether Sulfate (SLES), and Sodium Dodecyl Sulfate (SDS).[17–19] Research by Fazelian-Dehkordi et al. suggests that the use of SLES can effectively remove proteins and growth factors, thereby reducing osteoinductive potential.[20]

Sterilizing scaffold materials is an essential last measure to minimize the chances of spreading harmful germs. Different techniques can be used for sterilization, including ethylene oxide, gamma ray radiation, and thermal methods. Gamma ray radiation sterilization is cited as one factor contributing to a decrease in growth factor levels.[21,22]

The osteoinductive nature plays an important function in bone repair and is strongly related to growth factors. These growth factors work as signaling molecules near the defect site, allowing inflammatory and progenitor cells to move and begin the bone healing process. Some factors that play a significant role in the growth of bones during regeneration are Bone Morphogenetic Protein (BMP), Transforming Growth Factor Beta (TGF-β), Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), and Insulin-Like Growth Factors (IGF).[23] TGF-β and BMP are considered multifunctional growth

factors and are identified as the most critical regulators in all stages of bone regeneration. TGF-β1 plays a key role in initiating signaling for BMP synthesis by osteoprogenitor cells. Additionally, Bone Morphogenetic Protein-2 (BMP-2) has the capability to stimulate osteoblast activity and induce bone formation through bone-forming cells.

Wildemann et al. completed the first investigation to quantify growth factors in allografts.[21] The identified growth factors included BMP-2, BMP-4, IGF-I, VEGF, FGFa, TGF-β1, and PDGF. The quantification of growth factor levels was carried out through the extraction procedure using Guanidine HCl, proteinase, or collagenase methods followed by measurement with ELISA. The Guanidine HCl method was chosen due to its ability to extract a higher amount of proteins, including growth factors. Research on bovine bone processed by demineralization and decellularization or native bone decellularized ECM (bdECM) by Lee et al. reported higher levels of BMP-2 and BMP-7 compared to bone powder and Demineralized Bone Matrix (DBM).[24]

Variations in processing techniques, protocols, chemical additives, and gamma ray sterilization can lead to a decrease in growth factor content, therefore reducing osteoinductive potential. Given the background mentioned above, there is a need for further research to measure and compare growth factor levels in cancellous bovine bone that has undergone freeze-drying and decellularization processes.

MATERIAL AND METHODS

This research is an analytical observational study. The research design used in this study is cross-sectional, which is a data collection method conducted at a single point in time on the entire treatment group. The subjects in this study were divided into three treatment groups. The control group consisted of fresh cancellous bovine bone, which served as a natural reference standard. The first group used Freeze Dried Bovine Bone (FDBB) xenogenic scaffold, while the second group used Decellularized Freeze Dried Bovine Bone (dc-FDBB) xenogenic scaffold. TGF-β1 and BMP-2 levels have been measured in each group using a minimum sample size, using the formula proposed by Lemeshow et al.[25] In this study 6 samples will be used. The variables used in this study consist of independent variables, dependent variables, and controlled variables. The independent variable in this study is the type of bovine bone scaffold used, namely Freeze-Dried Bovine Bone (FDBB) and Decellularized Freeze-Dried Bovine Bone (dc-FDBB). The dependent variables in this study are the levels of TGF-β1 and BMP-2. Meanwhile, several variables were controlled to maintain the consistency and validity of the research results, such as the sterilization process carried out using 25 kGy gamma ray radiation, as well as the uniform protein extraction method using Guanidine HCl solution.

An ethical clearance certificate of approval for the study was issued by the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia, with a reference number of 814/HRECC.FODM/VII/2023. The extraction of growth factors using the Guanidine HCl method and quantification of growth factors by ELISA examination were performed at the Biochemistry Laboratory, Faculty of Medicine, Brawijaya University, Malang. The equipment used in this research included microcentrifuge tubes, centrifuge, cooling machine, plates or standard wells, single or multi-channel pipettes, containers, and a microplate reader with a 450 ± 10 nm filter. The materials used in this research were 4M Guanidine HCl solution, 50 mM EDTA solution, Tris solution pH 7.4, 5 mM Benzamidine-HCl solution, 1 mM Phenylmethylsulfonyl solution, 0.1 mM Aminocaproic acid solution, gloves, distilled water, TGF-β1 ELISA kit (SEA124Bo, Cloud-Clone Corp., TX, USA), BMP-2 ELISA kit (SEA013Bo, Cloud-Clone Corp., TX, USA), absorbent paper, 0.01mol/L Phosphate Buffered Saline (PBS) pH 7.0-7.2, Fresh Bovine Cancellous Bone samples, FDBB scaffold, and dc-FDBB scaffold.

Freeze-Dried Bovine Bone (FDBB) is a scaffold produced by the National Research and Innovation Agency (BRIN), derived from cancellous part of bovine femur bone cut into 1x1x1 cm size. The bone is first cleaned using high pressure water, then soaked in a mixture of chloroform and methanol in equal proportions for three hours. Afteward, the bone is rinsed using sterile distilled water and soaked again in a 3% H_2O_2 solution for three hours, followed by another rinse using sterile distilled water. The next step is freeze-drying at -80°C, then dried using a lyophilizer machine until the water content is below 10%, with a drying duration of 15 hours. The scaffold is then packaged and sterilized using 25 kGy of gamma ray radiation. Decellularized Freeze-Dried Bovine Bone (dc-FDBB) is also a scaffold produced by BRIN with similar raw materials and size, which is cancellous bovine femur bones sized at 1x1x1 cm. After initial cleaning with high-pressure water and soaking in a chloroform-methanol mixture for three hours, the bones are cleaned with sterile distilled water. The next process involves soaking in a 3% H_2O_2 solution containing the ionic surfactant Sodium Lauryl Ether Sulfate (SLES), assisted by the use of a sonicator for three hours, followed by rinsing. The bones then undergo freeze-drying at -80°C and dried using a lyophilizer for 15 hours until the water content is less than 10%. Once dried, the scaffold is packaged and sterilized with 25 kGy gamma ray radiation. The level of TGF-β1 is defined as the concentration of TGF-β1 protein extracted from Fresh Cancellous Bovine Bone, FDBB, or dc-FDBB using the

Guanidine HCl (GndHCl) extraction method. The supernatant extract from each type of graft is measured using a sandwich ELISA method at a wavelength of 450 nm, with the measurement results expressed in pg/mL units. Similarly, the level of BMP-2 refers to the concentration of BMP-2 protein extracted from all three types of graft using the same method, and measured with a similar ELISA procedure, with the results also expressed in pg/mL units. The ELISA kits used for analyzing the levels of TGF-β1 and BMP-2 were also standardized, with each using the TGF-β1 (SEA124Bo) and BMP-2 (SEA013Bo) ELISA kits, both produced by Cloud-Clone Corp., TX, USA.

Guanidine HCl Extraction Method

FDBB, dc-FDBB, and fresh cancellous bovine bone were all subjected to the extraction procedure utilizing the Guanidine HCl technique. A 1.5 ml centrifuge tube containing 30 mg of fresh bovine cancellous bone sample was used. The centrifuge tube was filled with 1.9 ml of 4M Guanidine HCl solution, 50 mM EDTA, 5 mM benzamidine-HCl solution plus 50 mL Tris pH 7.4, and 0.1 mM aminocaproic acid solution. Following the mixing of these solutions, the sample was dialyzed for 24 hours at 4°C using distilled water. Four dialysis sessions were conducted. The material was centrifuged at 12,000 rpm to create supernatant once extraction was finished. The same extraction procedure was applied on FDBB and dc-FDBB scaffolds.

TGF-β1 and BMP-2 Quantification

In this study, the measurement of growth factor levels was carried out using the ELISA sandwich method following the protocol provided by the ELISA kit used. The ELISA kit TGF-β1 (SEA124Bo, 48 test, Cloud-Clone Corp., TX, USA) was utilized for measuring TGF-β1, while the ELISA kit BMP-2 (SEA013Bo, 48 test, Cloud-Clone Corp., TX, USA) was used for measuring BMP-2.

The procedure begins by determining the wells to be used for standard dilutions, blanks, and samples. Seven wells are prepared for standards and one well for the blank. Each well is then filled with 100 µL of standard solution, blank, or sample, then covered using a plate sealer and incubated for one hour at 37°C. After the first incubation, the liquid in each well is discarded without washing. Next, 100 µL of the prepared Detection Reagent A solution is added to each well. The plate is covered again and incubated for one hour at 37°C.

The washing process is performed by adding 350 µL of 1× wash buffer solution to each well using a multi-channel pipette and allowing it to stand for 1–2 minutes. After that, the solution is discarded and the plate is placed on absorbent paper. This washing step is repeated three times. After the final wash, remaining wash buffer is removed by aspiration or pouring, then the plate is inverted and placed back on absorbent paper. The next step is adding 100 µL of Detection Reagent B to each well, then the plate is covered again and incubated for 30 minutes at 37°C. After incubation, the washing process is repeated five times as in the previous step. Next, 90 µL of substrate solution is added to each well. The plate is then covered with a new plate sealer and incubated for 10 to 20 minutes at 37°C, noting not to exceed 30 minutes and protected from light. During this process, the liquid in the wells will change color to blue. After that, 50 µL of stop solution is added to each well, which will cause the liquid to change color to yellow. The plate is then tapped gently to mix the well contents evenly. Before reading, the bottom surface of the plate is cleaned of water droplets and fingerprints, and it is ensured that there are no air bubbles on the liquid surface. Results are read immediately using a microplate reader at a wavelength of 450 nm.

Statistical Analysis

The research variables were analyzed using several stages of statistical methods. Initially, a descriptive data analysis was conducted to provide an overall picture of the obtained data. Subsequently, normality testing was performed using the Shapiro-Wilk test, where a p-value greater than 0.05 indicates that the data is normally distributed. In the next step, homogeneity of variance was assessed using the Levene Test. If the significance value (p) is greater than 0.05, then the data is considered homogeneous or from populations with similar variances. For data that meet the assumptions of normality and homogeneity, a comparative analysis was conducted using One Way ANOVA. The results of the ANOVA test with a p-value < 0.05 indicate rejection of the null hypothesis (H0), meaning there is a difference in growth factor levels between Fresh Bovine Cancellous Bone, FDBB, and dc-FDBB. Differences in means between groups were further analyzed using the Post Hoc Tukey test, and if $p < 0.05$, significant differences between groups were identified. However, if the data is normally distributed but not homogenous, the Kruskal-Wallis test is used as an alternative. Similar to before, a p-value < 0.05 indicates significant differences between groups. To identify which pairs of groups are significantly different, advanced testing is conducted using the Mann-Whitney test. A p-value < 0.05 in this test also indicates significant differences between the two compared groups.

RESULTS

Findings from the study revealed that the levels of Transforming Growth Factor-Beta 1 (TGF-β1) were proportional to absorbance or optical density.

In the Fresh Cancellous Bovine Bone group, the TGF-β1 levels ranged from 15,528 pg/mL to 18,444 pg/mL with a mean of 16,453 pg/mL. In the FDBB group, the TGF-β1 levels ranged from 4,000 pg/mL to 17,750 pg/mL with a mean of 9,300 pg/mL. In the dc-FDBB group, the TGF-β1 levels ranged from 1,639 pg/mL to 9,556 pg/mL with a mean of 6,291 pg/mL. The findings suggest that the Fresh Cancellous Bovine Bone group has elevated levels of TGF-β1 compared to the FDBB and dc-FDBB groups.

The quantity of BMP-2 levels is equivalent to optical density or absorbance. The BMP-2 levels in the Fresh Cancellous Bovine Bone group averaged 10,181 pg/mL, with a range of 7,518 pg/mL to 11,714 pg/mL. The BMP-2 levels in the FDBB group averaged 7,086 pg/mL, with a range of 5,286 pg/mL to 8,589 pg/mL. The BMP-2 levels in the dc-FDBB group averaged 5,672 pg/mL and varied from 1,446 pg/mL to 9,036 pg/mL. According to these findings, the Fresh Cancellous Bovine Bone group had greater BMP-2 levels than the FDBB and dc-FDBB groups. The data shows that the test results align with the initial research hypothesis, stating that the TGF-β1 and BMP-2 levels in the Fresh Cancellous Bovine Bone group are higher than those in the FDBB and dc-FDBB groups.

Table 1. ELISA test results of TGF-β1 and BMP-2 levels

	N	Average	Standard Deviation	Minimum	Maximum
TGF-β1					
Fresh Cancellous Bovine Bone	6	16,453	1,048	15,528	18,444
FDBB	6	9,300	5,057	4,000	17,750
dc-FDBB	6	6,291	3,081	1,639	9,556
BMP-2					
Fresh Cancellous Bovine Bone	6	10,181	1,469	7,518	11,714
FDBB	6	7,086	1,262	5,286	8,589
dc-FDBB	6	5,672	2,695	1,446	9,036

ELISA Test Analysis of TGF-β1 and BMP-2 levels

1. Normality Test (Shapiro-Wilk)

The normality test is utilized to determine whether the gathered study data follows a normal distribution. The Shapiro-Wilk test is chosen for the normality test. Table 2 displays the outcomes of the normality test for TGF-β1 and BMP-2 levels as assessed by the Shapiro-Wilk test. The normality test results on the levels of TGF-β1 show a p-value of 0.084 for the Fresh Cancellous Bovine Bone group, p = 0.519 for the FDBB group, and p = 0.625 for the dc-FDBB group. Meanwhile, the normality test results on the levels of BMP-2 show a p-value of 0.335 for the Fresh Cancellous Bovine Bone group, p = 0.747 for the FDBB group, and p = 0.851 for the dc-FDBB group. From the data obtained, it is found that for all groups, the sig value is greater than 0.05 so H0 is accepted, indicating that the data in all groups are normally distributed.

Table 2. Normality test results of TGF-β1 and BMP-2 levels

Test Type	Test Name	Variable	P value (sig.)
Normality Test	Shapiro-Wilk	**TGF-β1**	
		Fresh Cancellous Bovine Bone	0,084*
		FDBB	0,519*
		dc-FDBB	0,625*
		BMP-2	
		Fresh Cancellous Bovine Bone	0,355*
		FDBB	0,747*
		dc-FDBB	0,851*

*) *p-value* > 0.05 indicates the data is normally distributed

2. Homogeneity Test (Levene's Test)

The homogeneity test checks if multiple sample data sets come from populations with equal variances, after confirming that the data follows a normal distribution. The results of the homogeneity test are shown in Table 3, using the Levene's Test Method. The Levene's Test reveals a p value of 0.024 (p < 0.05) for TGF-β1 levels, suggesting that the variance is not consistent. The findings of the Levene's Test on BMP-2 levels reveal a p value = 0.222, where the result is p> 0.05, indicating an equality of variance between groups.

Table 3. Homogeneity test results of TGF-β1 and BMP-2 levels

Test Type	Test Name	Variable	P value (sig.)
Homogeneity Test	*Levene's Test*	TGF-β1 levels	0,024
		BMP-2 levels	0,222*

*p value > 0,05 indicates similarity of variance between groups (homogeneous)

3. Comparison test

a) Comparative test of TGF-β1 levels

Since the Shapiro-Wilk normality test indicated that the data distribution was normal but the Levene's test homogeneity revealed that the data was not homogenous, the Kruskal-Wallis test was applied in the present study. The Kruskal-Wallis test on TGF-β1 results in a p value of 0.05, which means we can conclude that every group differs significantly.

Table 4. Comparative test results of TGF-β1 levels

Group		*Kruskal-Wallis*	*Mann Whitney*
Fresh Cancellous Bovine Bone	FDBB	0,01*	0,037*
	dc-FDBB		0,004*
FDBB	*Fresh Cancellous Bovine Bone*		0,037*
	dc-FDBB		0,378
dc-FDBB	*Fresh Cancellous Bovine Bone*		0,004*
	FDBB		0,378

*) *p-value* < 0.05 there is a significant difference in the comparative test

The Mann Whitney test is utilized to determine any notable variances between groups during post hoc analysis. According to the test results, it is evident that there is no substantial distinction between the FDBB and dc-FDBB groups since their p values are 0.378 and >0.05. The p-values for the Fresh Cancellous Bovine Bone and FDBB groups are 0.037 and 0.004, respectively, according to the Fresh Cancellous Bovine Bone and dc-FDBB groups. The Fresh Cancellous Bovine Bone and FDBB groups and the Fresh Cancellous Bovine Bone and dc-FDBB groups differ significantly, as indicated by the p value <0.05.

b) Comparative test of BMP-2 levels

Due to the results of the Shapiro-Wilk normality test suggesting a normal and homogenous distribution of the data, the analysis for BMP-2 levels utilized the One Way Anova test. The results of the One Way Anova test revealed significant variances in BMP-2 levels among the three groups, with a p value of 0.003 or p < 0.05.

Table 5. Comparative test results of BMP-2 levels

	Group	*One Way Anova*	*Mann Whitney*
Fresh Cancellous Bovine Bone	FDBB	0,03*	0,034*
	dc-FDBB		0,003*
FDBB	*Fresh Cancellous Bovine Bone*		0,034*
	dc-FDBB		0,429
dc-FDBB	*Fresh Cancellous Bovine Bone*		0,003*
	FDBB		0,429

*) *p-value* < 0.05 there is a significant difference in the comparative test

To find out whether groups differ significantly, the Tukey Test is used for post hoc or further testing. There is no significant difference between the FDBB and dc-FDBB groups, as shown by the Tukey Test results, which show that the p value for both groups is 0.429 or greater than 0.05. P values for the Fresh Cancellous Bovine Bone and FDBB groups were 0.034 and 0.003, respectively, as was the case for the Fresh Cancellous Bovine Bone and dc-FDBB groups. The Fresh Cancellous Bovine Bone and FDBB groups and the Fresh Cancellous Bovine Bone and dc-FDBB groups differ significantly, as indicated by the p value <0.05.

4.DISCUSSION

The objective of the current research was to examine the levels of TGF-β1 and BMP-2 in Fresh Cancellous Bovine Bone, FDBB scaffolding, and dc-FDBB scaffolding. Bovine bone is structurally and functionally similar to human bone so it was chosen in this study to observe the difference in the decrease in growth factor levels after freeze-dried and desulphurization processing. Fresh Cancellous Bovine Bone was used as the control group. The average content of TGF-β1 and BMP-2 were detected more in Fresh Cancellous Bovine Bone than FDBB and dc-FDBB. Research showing that freeze-dried procedures, desellularization processes carried out involving exposure to chemical and biological agents, and sterilization with gamma radiation has the potential to lower the concentration of osteogenic growth factors.[13,26–31]

In this investigation, the application of H_2O_2 solution for 3 hours causes the levels of TGF-β1 and BMP-2 to drop in FDBB and dc-FDBB. This corresponds with studies on human cortical bone effects of H_2O_2.[15] According to the previous discoveries, H_2O_2 can interfere with the osteoinduction capacity of graft materials as well as BMP.[15] As a chemical oxidant, H_2O_2 can influence the proteins in bones. Using H_2O_2 could help to lower the osteoinductivity of a transplant and BMP. With increasing time, statistically H_2O_2 produces a notable drop in osteoinductivity. Previous research show that the osteoinductivity score statistically dropped five hours after immersion in H_2O_2.[15] Osteoinductivity was not much changed by the one hour H_2O_2 immersion cleaning process. This is the reason the phase of the cleaning process with H_2O_2 is preferably confined to immersion for 1 hour.

Another investigation conducted by Qing et al. evaluated BMP-2, which was selected as an indicator to evaluate the osteoinductive capabilities of materials and to demonstrate the efficacy of H_2O_2.[32] Two graft materials were employed in his research: Decellularized and Demineralized Bone Matrices (DCDBM) and H_2O_2 -treated DCDBM (HPTBM), which is DCDBM that has been immersed in a 3% H_2O_2 solution for 12 hours. Whereas DCDBM showed positive staining, IHC staining results revealed negative staining for BMP-2 in HPTBM. This work also included IHC staining for TGF-β1 and VEGF in the two materials; the findings were negative staining. This suggests that the growth factor content in HPTBM was absent, thereby explaining the variations in osteoinductive activity of the two materials. This points out that a soaking period longer than one hour and the use of H_2O_2 itself may possibly lower growth factor levels.

Decellularization is another process that might cause the drop in growth factor levels.[33]

This work acquired the decellularization technique to generate dc-FDBB scaffold by immersion in a 3% H2O2 solution augmented with SLES. This is comparable with studies by Dehkordi et al., which reveal that SLES can eliminate glycosaminoglycan (GAG), growth factors, and protein, content. An ECM component, GAG is involved in several biological events including cell adhesion, control of cell growth, and cell proliferation. SDS can lyse cells effectively, denature proteins, degrade collagen, and lower GAG in the ECM.[34] Moreover, SDS's ionic character causes it not to be totally eliminated from the tissue, hence careful washing is necessary. Triton X-100 eliminates core content from cells and raises their permeability in tissue. Measuring by ELISA assay, research by Das et al. found TGF-β1 both before and after the decellularization process.[35] In their work, Triton X-100 treated samples indicated either a 47% drop in TGF-β1 levels or higher loss of growth factor levels. This is due to the Triton X-100 has a strong non-ionic character. Ma et al. conducted research showing that compared to SDS, SLES for decellularization supports tissue architecture, has reduced toxicity, and preserves ECM characteristics.[36] The group treated with SLES produced ordered collagen fiber orientation akin to that of undamaged bone orientation. Treatment with SLES also produced notably reduced DNA content. Accordingly, every suggested approach should strike a balance between removing cells and preserving ECM content.[37–39]

The last stage of this work was sterilizing FDBB scaffold and dc-FDBB under 25 kGy gamma radiation and packing. This level seeks to lower storage resistance, ease of transit, and transmission risk of infectious pathogens. Several investigations confirming that streralization under gamma radiation is one of the elements lowering growth factor levels support the conclusions of this work. Gamma radiation reduces collagen fibers and growth factors, therefore impairing the mechanical quality and biological properties of the scaffold in the form of a degradation of bone matrix. Al Kayal et al.[40] have reported a drop in BMP-2 and BMP-7 concentrations in DBM sterilized under 25 kGy gamma radiation.[41–44] Gamma radiation disrupts the polypeptide chains, thereby damaging BMPs and so reducing the osteoinductivity of the bone graft.[45] Some research findings, on the physical and biological characteristics of allograft tissue as in the laboratory and clinical study[46], contradict this by stating that radiation sterilization with doses up to 25 kGy has no negative effect on these aspects.[42,47]

The comparison test in this study revealed significant differences between the Fresh Cancellous Bovine Bone and FDBB groups, as well as the Fresh Cancellous Bovine Bone and dc-FDBB groups, but no significant differences between the FDBB and dc-FDBB groups.

This is so because FDBB and dc-FDBB were subjected to chemical and biological agents as well as gamma radiation sterilization, therefore lowering the concentration of growth factors. The inclusion of SLES surfactant in dc-FDBB serves to remove cells and DNA from the tissue but still preserves proteins, so there is a negligible difference between the FDBB and dc-FDBB groups. The objective is to eradicate the immunogenicity and disease transmission risks that FDBB continues to possess. Though it is not totally eliminated, the overall protein count is dropped. Strong anionic detergent with more biodegradability, SLES protects ECM proteins and microarchitecture better and has softer chemical characteristics, which will be crucial for cell implantation.[22,39] In addition, dc-FDBB is also preferable due to its potential osteoconducting, osteoinductive, and non-antigenic properties as a biocompatible bone graft substitute.

The study's limitation is that it did not examine the factors of the scaffold preparation protocol that influence growth factor levels. Additionally, this investigation assessed the concentrations of growth factors TGF-β1 and BMP-2, despite the existence of several additional growth factors that contribute to bone regeneration.

CONCLUSION

Fresh Cancellous Bovine Bone exhibited the highest concentrations of TGF-β1, followed closely by Freeze-Dried Bovine Bone (FDBB), with de-cellularized Freeze-Dried Bovine Bone (dc-FDBB) displaying the lowest levels. A similar trend was observed for BMP-2 levels, which were also highest in Fresh Cancellous Bovine Bone, followed by FDBB, and lowest in dc-FDBB. Although lower concentrations of TGF-β1 and BMP-2 were observed in bovine bone subjected to freeze-drying and decellularization, no statistically significant difference was found in the levels of TGF-β1 and BMP-2 between FDBB and dc-FDBB.

In order to prevent disruption of growth factor components, the authors recommend a one-hour H_2O_2 washing procedure and further investigation into the factors that influence growth factor levels in the scaffold production protocol. Additionally, further studies assessing other growth factors involved in bone regeneration are encouraged to enhance scaffold material preparation techniques for maxillofacial bone regeneration and provide supporting data for scaffold development.

DECLARATIONS

Ethics approval and consent to participate

Ethical approval was obtained from Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia (Reference: 814/HRECC.FODM/VII/2023).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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