

DOI: 10.58240/1829006X-2025.21.6-158



ORIGINAL RESEARCH

COMPARISON OF SERUM CYTOKINES IL-6, TNF- α , AND IL-10 AFTER SUBCUTANEOUS IMPLANTATION OF DECELLULARIZED AND NON DECELLULARIZED FREEZE DRIED BOVINE BONE SCAFFOLD (In vivo laboratory research on Rattus Novergicus)

Kharisma Nisa¹, David Buntoro Kamadjaja^{2*}, Ni Putu Mira Sumarta²

1. Department of Clinical Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

2. Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

***Corresponding Author:** David Buntoro Kamadjaja, Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga. Jl. Prof. Dr. Moestopo No. 47 Surabaya 60132 – Indonesia. Email ID: david-b-k@fkg.unair.ac.id

Received: Jun 7, 2025; Accepted: Jun 28, 2025; Published: Jul 15, 2025

ABSTRACT

Background: The xenogeneic scaffold such as FDBB is considered immunogenic due to the presence of xenogeneic cell residue. A decellularization process is added to FDBB to reduce the inflammatory response.

Objectives: The objective of this study is to evaluate the efficacy of dc-FDBB in reducing the host inflammatory response after implantation, compared to DBBM as the gold standard scaffold with renowned biocompatibility.

Method: Sixty Rattus Novergicus were divided into four groups, subcutaneously implanted FDBB, dc-FDBB, DBBM scaffolds and one group is sham-operated. On the 2nd, 7th and 14th postoperative days, a thoracotomy was performed and 1.5 ml of intra-cardiac blood was taken for ELISA test. Statistical tests were carried out by testing the normality of the data (Shapiro-Wilk) and homogeneity (Levene), then a comparative test was carried out with the Analysis of Variance (ANOVA) on data that was normally distributed and homogeneous, on data that was not normally distributed or not homogeneous, a comparison test with the Kruskal Wallis test, followed by the Post Hoc Tuckey HSD test ($p < 0.05$).

Results: Statistical analysis on days 2, 7 and 14 on levels of IL-6 ($p = 0.530$, $p = 0.279$, $p = 0.790$), TNF- α ($p = 0.250$, $p = 0.009$, $p = 0.324$), and IL -10 ($p = 0.743$, $p = 0.349$, $p = 0.374$ as a whole showed the result ($p > 0.05$), there was no significant difference between all groups. Results of analysis of TNF- α day 7 ($p = 0.009$) with ($p > 0.05$) showed that there was a difference, and continued with the post hoc test with the result $p = 0.005$, there was a difference between the controls compared to the dc-FDBB.

Conclusion: There was no significant difference in serum levels of IL-6, TNF- α , and IL-10 after FDBB and dc-FDBB implantation.

Keywords: Freeze Dried Bovine Bone, Decellularized, Systemic Inflammatory Response, Subcutaneous Implantation

INTRODUCTION

Reconstructing bone defects in the mandibular bone caused by trauma, neoplasia, infection, and degenerative diseases is essential in restoring jaw function and quality of life for patients. Extensive defects in the maxillofacial region can lead to disturbances in both aesthetics and stomatognathic function for patients.¹ Autologous bone graft continues to be the gold standard in the management of defects due to its osteogenic, osteoinductive, and

osteoconductive properties. However, it is limited by its availability and can result in defects at the donor site and increased morbidity.²

Tissue engineering is a technology in the field of medicine that is currently being developed with the basic concept of combining cells, scaffolds, and growth factors. The current challenge in bone tissue engineering is to create an ideal biomaterial that is both mechanically and biologically suitable, enabling the formation of bone tissue with good vascular support for defect reconstruction.^{3,4}

The use of xenogenic materials is preferred due to their lack of donor site morbidity and high bioavailability, making them easily accessible. The immune response of the recipient to the immunogenic components of xenogenic tissues poses a critical barrier in scaffold applications. Unwanted immune responses can lead to early resorption, fibrosis at the implant site, implant rejection reactions, and ultimately failure.^{5,6}

Freeze-Dried Bovine Bone Scaffold (FDBB) is considered as an alternative in tissue engineering for the reconstruction of mandibular defects while retaining organic components.⁷ In a previous study by Montessory et al, 2022, FDBB scaffold showed some damaged osteocyte residues and no osteoblast cells were found, whereas dc-FDBB and DBBM scaffolds did not show any osteocyte or osteoblast residues. The average DNA concentrations of FDBB, dc-FDBB, and DBBM scaffolds were measured using a spectrophotometer, with mean values of 19.75 ng/ μ L, 16.84 ng/ μ L, and 8.72 ng/ μ L, respectively, indicating that FDBB is considered to contain higher DNA content.⁸

The process of decellularization (removal of remaining cells) is one strategy to eliminate antigenic and immunogenic properties on xenograft scaffolds. Decellularization process removes all cellular components from the original tissue while retaining the structure and composition of the Extra Cellular Matrix (ECM) as much as possible to achieve an osteoinductive scaffold.⁹ There are various methods of decellularization procedures, but a standardized decellularization process has not yet been patented due to the diverse characteristics and protocols used in different research groups.¹⁰ Decellularization is achieved chemically by using detergents such as Triton X-100, sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and ammonium hydroxide. Freezing/thawing cycles are a physical process that can also be used in the decellularization process. The combination of chemical and physical processes can enhance efficient decellularization results and preserve the ECM.^{7,11}

Deproteinized Bovine Bone Mineral (DBBM) has been used for a long time, but it has the disadvantage of having all the important organic components extracted during the manufacturing process, making it non-osteoinductive. Freeze Dried Bovine Bone (FDBB) was later developed as a good choice for bone grafting as it retains organic materials such as growth factors and other cellular components while maintaining inorganic materials.⁷

In vivo research has been employed in several studies to examine the body's response following the implantation of scaffolds.⁹ Subcutaneous implantation

in small animal models facilitates preclinical testing assessing immune reactivity and reseularization capacity quite effectively nowadays.¹² Subcutaneous implantation in mice gets studied extensively owing largely to its capacity for evaluating factors like potential sensitization and genotoxicity fairly accurately.¹²

Cytokine measurement in body fluids like serum offers a valid method for examining inflammatory responses over time amidst complex immune system interactions.¹³ This method is utilized to assess systemic reactions and the severity of inflammatory responses following the subcutaneous implantation of freeze-dried bovine bone scaffolds, both decellularized and non-decellularized.

Interleukin-6 (IL-6) is a proinflammatory cytokine initially identified as a stimulator of B cells. IL-6 has various functions related to the regulation and coordination of the immune system, metabolism, and nervous system¹⁴. TNF- α initiates an essential mediator cascade of inflammation that correlates with IL-1 β . TNF- α stimulates prostaglandin synthesis, resorption¹³, and regulates the differentiation and function of osteoblasts and osteoclasts.¹⁵ IL-10 is an anti-inflammatory cytokine, a multifunctional cytokine, a critical immune modulator, and plays a positive role in maintaining the balance of immune responses, dampening acute inflammatory feedback, and generating a negative feedback loop that reduces the release of inflammatory mediators.¹⁶ IL-6, TNF- α , and IL-10 in the context of the pro and anti-inflammatory actions of the immune system affect innate immunity and adaptive immunity to scaffold implantation, therefore necessitating in vivo studies to investigate the non-specific response post-implantation of freeze-dried bovine bone scaffolds, both decellularized and non-decellularized, by comparing serum levels of IL-6, TNF- α , and IL-10.

In this paper, analysis of serum IL-6 TNF- α and IL-10 levels following subcutaneous implantation of decellularized and non-decellularized freeze-dried bovine bone scaffolds was undertaken.

MATERIAL AND METHODS

This study represents an in vivo laboratory research. The research design employed was a post-test only control group design, comparing the serum levels of IL-6, TNF- α , and IL-10 in *Rattus norvegicus* on days 2, 7, and 14. The research groups were divided into 4 categories: Group 1 consisted of *Rattus norvegicus* that underwent the same surgery but were not implanted. Group 2 included *Rattus norvegicus* implanted with deproteinized bovine bone material scaffold. Group 3 comprised *Rattus norvegicus* implanted with freeze dried bovine bone xenograft scaffold subcutaneously. Group 4 consisted of *Rattus norvegicus* implanted with decellularization freeze dried bovine bone xenograft scaffold. Serum levels of IL-6, TNF- α , and IL-10 were measured at three different

time points for each group: on day 2, day 7, and day 14. Replication at each observation time was conducted with the appropriate minimum sample size calculated beforehand.

An ethical approval was granted by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine at Universitas Airlangga in Surabaya, Indonesia under Ethical Clearance Number: 2.KEH.125.09.2022 on September 20, 2022. All experiments on animals were carried out following the institution's protocols for animal welfare.

The sample size used in this study was calculated based on the calculation formula.¹⁷ In order to address the possibility of failure, the proportion of drop-outs from the sample was calculated using the formula: $n' = n + 10\% \text{ of } n$. It was found that $n'=4$. Therefore, there were 5 samples in each group of the study, multiplied by 4 groups, and then multiplied again by 3 observation times. The total number of observations was 60 observations in each kit. In this study, serum IL-6, TNF- α , and IL10 were observed, resulting in 180 observations.

In this study, the independent variables included three types of materials, namely deproteinized bovine bone mineral (DBBM) xenograft, freeze-dried bovine bone xenograft (FDBB) xenograft, and decellularized freeze-dried bovine bone xenograft (dc-FDBB) xenograft. The dependent variables measured were serum levels of IL-6, TNF- α , and IL-10, which were analyzed using the ELISA method. Additionally, the controlled variable in this study was the type of test animal, specifically *Rattus norvegicus* strain Wistar. The equipment utilized in this study includes a hair clipper, handle scalpel, curved clamp, needle holder, anatomical tweezers, surgical tweezers, suture scissors, 1cc syringe, rat blood storage media, rat ELISA kits for IL-6, TNF- α , and IL-10, as well as ELISA testing tools. The research materials consist of Scaffold DBBM, FDBB, dc-FDBB, ketamine, a 10% povidone iodine solution, No #15 blade, suturing needle, Silk 3-0 thread, and topical antibiotics.

RESULTS

Based on a study conducted during the period of November – December 2022, involving a sample size of 60 Wistar rats divided into 4 groups: subcutaneous FDBB scaffold, dc-FDBB, DBBM (positive control), and sham-operated (negative control). All experimental animals were in good health, with no signs of illness or disability, and there were no fatalities.

Serum IL-6 Levels Results

The serum IL-6 levels in this study can be seen in

Table 1. The data results show the highest mean in Group 4, which is the dc-FDBB implantation on day 7, at $12,612 \pm 2,143$ pg/ml. Whereas the lowest serum IL-6 level is found in Group 4, which is the dc-FDBB implantation on day 2, at $7,414 \pm 1,537$ pg/ml. In the control group, there is an increase in the mean IL-6 level from day 2 ($9,227 \pm 2,927$ pg/ml) to day 7 ($10,424 \pm 4,115$ pg/ml), and then a decrease on day 14 ($9,683 \pm 3,158$ pg/ml). In Group 2, which is the DBMM implantation, there is a consecutive decrease in the mean IL-6 level from day 2 ($9,873 \pm 2,442$ pg/ml) to day 7 ($9,098 \pm 2,077$ pg/ml), and day 14 ($8,527 \pm 2,556$ pg/ml). In the third group, which is the FDBB implantation, there is an increase in the mean IL-6 level from day 2 ($9,050 \pm 3,422$ pg/ml) to day 7 ($11,060 \pm 2,126$ pg/ml), and then a decrease on day 14 ($8,159 \pm 1,947$ pg/ml). In Group 4, which is the dc-FDBB implantation, there is an increase in the mean IL-6 level from day 2 ($7,414 \pm 1,537$ pg/ml) to day 7 ($12,612 \pm 2,143$ pg/ml), and then a decrease on day 14 ($8,444 \pm 2,354$ pg/ml).

Statistical testing was conducted with results as shown in Table 2. Normality tests using the Shapiro-Wilk test on days 2, 7, and 14 revealed significant values for the control group: $p = 0.119$, $p = 0.223$, $p = 0.61$; for DBBM: $p = 0.384$, $p = 0.908$, $p = 0.701$; for FDBB: $p = 0.111$, $p = 0.318$, $p = 0.533$; and for dc-FDBB: $p = 0.848$, $p = 0.617$, $p = 0.787$, all with $p > 0.05$ indicating normal data distribution. Subsequent homogeneity testing using Levene's test showed insignificant values on days 2, 7, and 14: $p = 0.218$, $p = 0.69$, $p = 0.761$, with $p > 0.05$ indicating homogeneous data. This was followed by One-Way ANOVA testing, which yielded results as presented in Table 2, with p values of 0.530 on day 2, 0.279 on day 7, and 0.790 on day 14, indicating no significant differences ($p > 0.05$) among groups on days 2, 7, and 14.

Serum TNF- α Levels Results

The serum TNF- α levels in this study can be observed in Table 3. The data results indicate the highest mean in group 4, which received dc-FDBB implantation on day 2 ($62,164 \pm 12,732$ pg/ml). On the other hand, the lowest serum TNF- α levels were found in the control group on day 7 ($29,434 \pm 17,291$ pg/ml). In the control group, there was a decrease in the mean TNF- α levels from day 2 ($48,589 \pm 12,593$ pg/ml) to day 7 ($29,434 \pm 17,291$ pg/ml), followed by a slight increase on day 14 ($32,264 \pm 11,817$ pg/ml). In group 2, which received DBMM implantation, there was a consecutive decrease in mean TNF- α levels from day 2 ($52,424 \pm 13,999$ pg/ml) to day 7 ($47,171 \pm 14,018$ pg/ml), and day 14 ($37,239 \pm 15,428$ pg/ml). Group three, which received FDBB implantation, also showed a consecutive decrease in mean TNF- α levels from day 2 ($54,867 \pm 18,181$ pg/ml) to day 7 ($47,619 \pm 7,793$ pg/ml), and day 14 ($45,359 \pm 14,093$ pg/ml). Finally, in group 4, which received dc-FDBB implantation, there was a consecutive decrease in mean

TNF-α levels from day 2 (62,164 ± 12,732 pg/ml) to day 7 (59,414 ± 2,707 pg/ml), and day 14 (47,070 ± 14,069 pg/ml).

The normality test using Shapiro-Wilk on day 2 revealed significant values for the control group p = 0.055; DBBM p = 0.218; with p < 0.05 indicating a normal distribution of data. However, for the FDBB group p = 0.008; dc-FDBB p = 0.032, with p < 0.05 indicating that the data did not follow a normal distribution, thus necessitating the use of the Kruskal-Wallis test on day 2, yielding a significant value of p = 0.250 (p > 0.05), indicating no significant differences between groups.

On days 7 and 14, the normality test revealed non-significant values of p > 0.05, indicating a normal distribution of data. For the control group on days 7 and 14, the significant values were p = 0.277, p = 0.671; DBBM p = 0.183, p = 0.289; FDBB p = 0.618, p = 0.216; and dc-FDBB p = 0.453, p = 0.394, all with p > 0.05 indicating a normal distribution of data. Subsequently, the homogeneity test using Lavene’s test yielded non-significant values on days 7 and 14, with p = 0.213, p = 0.776 (p > 0.05), indicating homogeneity, followed by the One-Way Anova test.

On day 7, a significant value of p = 0.009 (p < 0.05) indicated significant differences between groups. However, on day 14, a non-significant value of p = 0.324 (p > 0.05) indicated no significant differences between groups (Table 4).

The Post Hoc Test or Multiple Comparison, using Tukey HSD with a result of p = 0.005 (p < 0.05), indicates a significant difference in the head-to-head comparison between the dc-FDBB group and the control group (see Table 5).

Serum IL-10 Levels Results

The average serum IL-10 levels in this study showed the highest mean in the control group on day 7 (17,748 ± 23,168 pg/ml). Conversely, the lowest serum IL-10 levels were found in group 4, which underwent dc-FDBB implantation on day 14 (2,986 ± 1,983 pg/ml). In the control group, there was an increase in the average IL-10 levels from day 2 (5,532 ± 3,318 pg/ml) to day 7 (17,748 ± 23,168 pg/ml), followed by a decrease on day 14 (6,769 ± 2,844 pg/ml). For group

2, which received DBMM implantation, the mean IL-10 levels decreased from day 2 (4,892 ± 3,424 pg/ml) to day 7 (3,593 ± 2,464 pg/ml), and then showed a slight increase on day 14 (3,645 ± 2,563 pg/ml). In group 3, undergoing FDBB implantation, the mean IL-10 levels decreased from day 2 (8,971 ± 12,239 pg/ml) to day 7 (7,005 ± 1,761 pg/ml), and further decreased on day 14 (4,383 ± 4,678 pg/ml). A similar pattern was observed in group 4 (dcFDBB), where the mean IL-10 levels decreased from day 2 (9,506 ± 6,122 pg/ml) to day 7 (7,036 ± 5,676 pg/ml), and further decreased on day 14 (2,986 ± 1,983 pg/ml) (Table 6).

The statistical test results for IL-10 levels based on normality testing using the Saphiro-Wilk test on days 2, 7, and 14 showed that on day 2, the data in the FDBB group was found to be non-normal with a p-value of 0.040 (p < 0.05), while the control group had a p-value of 0.427, DBBM group had a p-value of 0.679, and dc-FDBB group had a p-value of 0.716, all of which were considered normal as the p-values were > 0.05. On days 7 and 14, the significance values for the control group were p = 0.364 and p = 0.359, for DBBM group p = 0.216 and p = 0.575, for FDBB group p = 0.652 and p = 0.237, and for dc-FDBB group p = 0.273 and p = 0.446, all of which were > 0.05, indicating that the data from all groups on days 7 and 14 were normally distributed. Further, homogeneity testing using Lavene’s test showed significant values on days 7 and 14, p = 0.160 and p = 0.098, respectively, both of which were > 0.05, indicating homogeneous data. Comparison tests between groups were then conducted using the Kruskal Wallis test and One Way ANOVA on days 7 and 14, yielding p-values of 0.743, 0.349, and 0.274 on days 2, 7, and 14, respectively, all of which were > 0.05, indicating no differences between groups on days 2, 7, and 14.

Table 1. Data Description of IL-6 data; mean & SD (pg/ml) between groups and between observation times

Group	N	Days 2		Days 7		Days 14	
		Mean	SD	Mean	SD	Mean	SD
Control	5	9,227	2,927	10,424	4,115	9,683	3,158
DBBM	5	9,873	2,442	9,098	2,077	8,527	2,556
FDBB	5	9,050	3,422	11,060	2,126	8,159	1,947
dc-FDBB	5	7,414	1,537	12,612	2,143	8,444	2,354

Table 1. Data Description of IL-6 data; mean & SD (pg/ml) between groups and between observation times

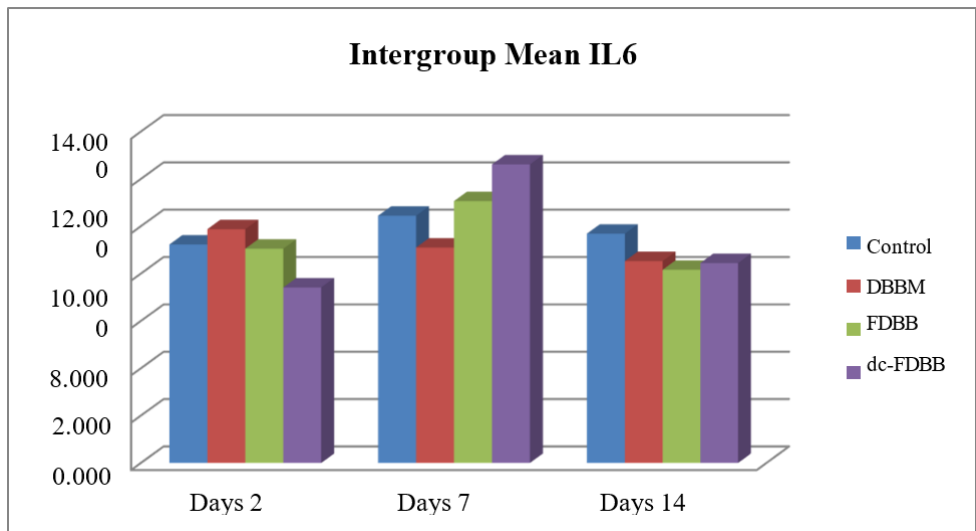


Figure 1. Graph of mean serum IL-6 levels between groups

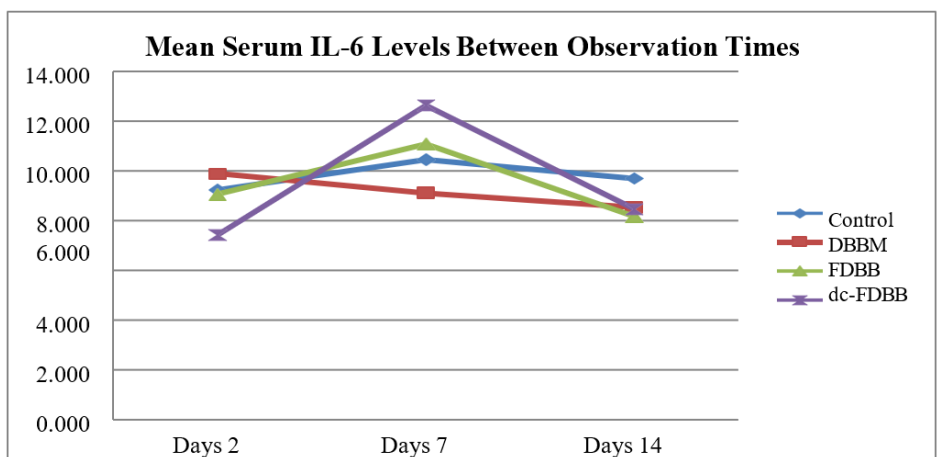


Figure 2. Graph of mean serum IL-6 levels between observation times

Table 2. Statistical analysis results of IL-6 data (tests for normality, Levene Test for homogeneity, and One Way Anova for differences between observations on days 2, 7, and 14)

Time	Group	Mean ± SD (pg/ml)	Saphiro-wilk (p)*	Lavene's test (p)*	One Way Anova (p)**
Day 2	Control	9,227 ± 2,927	0,119*	0,218*	0,530
	DBBM	9,873 ± 2,442	0,384*		
	FDBB	9,050 ± 3,422	0,111*		
	dc-FDBB	7,414 ± 1,537	0,848*		
Day 7	Control	10,424 ± 4,115	0,223*	0,069*	0,279
	DBBM	9,098 ± 2,077	0,908*		
	FDBB	11,060 ± 2,126	0,318*		
	dc-FDBB	12,612 ± 2,143	0,617*		
Day 14	Control	9,683 ± 3,158	0,061*	0,761*	0,790
	DBBM	8,527 ± 2,556	0,701*		
	FDBB	8,159 ± 1,947	0,533*		
	dc-FDBB	8,444 ± 2,354	0,787*		

*) p-value > 0.05 normal/homogeneous significant in normality test/homogeneity test;

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

Table 3. Description of TNF- α data; mean & SD (pg/ml) between groups and between observation times

Group	N	Days 2		Days 7		Days 14	
		Mean	SD	Mean	Mean	SD	Mean
Control	5	48,589	12,593	29,434	17,291	32,264	11,817
DBBM	5	52,424	13,999	47,171	14,018	37,239	15,428
FDBB	5	54,867	18,181	47,619	7,793	45,359	14,093
dc-FDBB	5	62,164	12,732	59,414	2,707	47,070	14,069

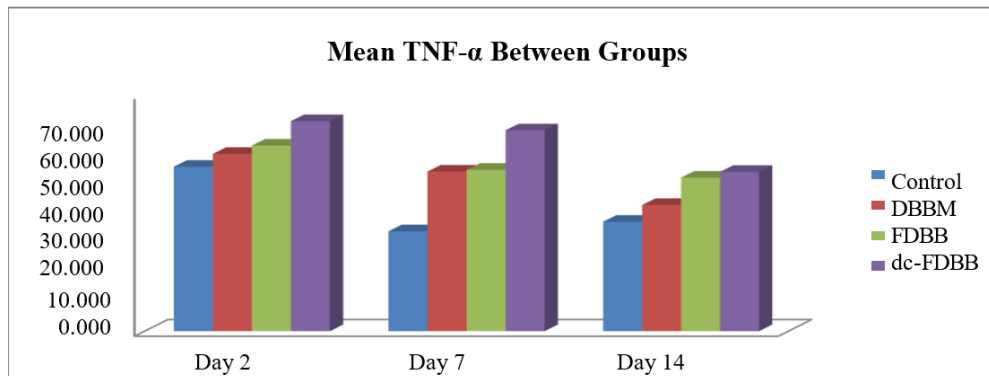


Figure 3. Graph of mean serum TNF- α levels

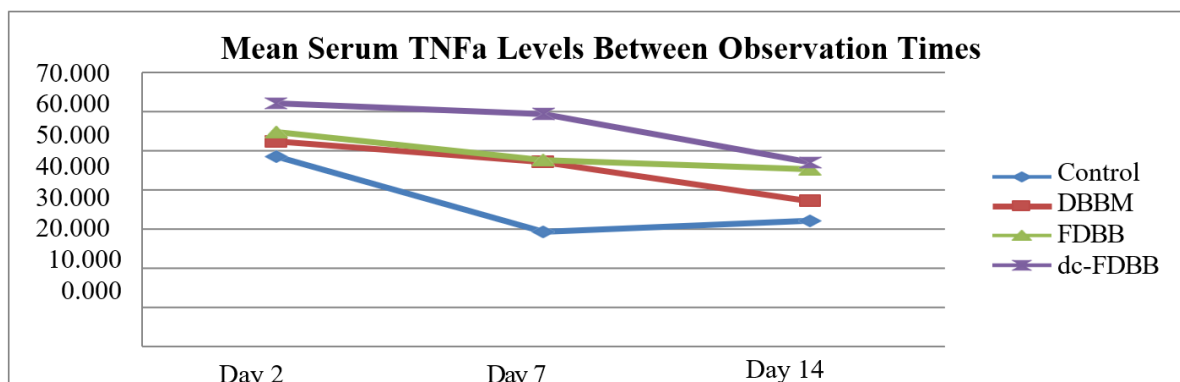


Figure 4. Graph of Mean Serum TNF- α Levels between observation times

Table 4. Results of statistical analysis of TNF- α data (normality test, Levene Test homogeneity test, and One Way Anova/Kruskall Wallis test) between observation times on days 2, 7, 14)

Time	Group	Mean \pm SD (pg/ml)	Saphiro-wilk (p)*	Lavene's test (p)*	One Way Anova (p)**	Kruskall Wallis (p)**
Day 2	Control	48,589 \pm 12,593	0,055*	-	-	0,250
	DBBM	52,424 \pm 13,999	0,218*			
	FDBB	54,867 \pm 18,181	0,008			
	dc-FDBB	62,164 \pm 12,732	0,032			
Day 7	Control	29,434 \pm 17,291	0,277*	0,213	0,009**	-
	DBBM	47,171 \pm 14,018	0,183*			
	FDBB	47,619 \pm 7,793	0,618*			
	dc-FDBB	59,414 \pm 2,707	0,453*			
Day 14	Control	32,264 \pm 11,817	0,671*	0,776	0,324	-
	DBBM	37,239 \pm 15,428	0,289*			
	FDBB	45,359 \pm 14,093	0,216*			
	dc-FDBB	47,070 \pm 14,069	0,394*			

*) p-value > 0.05 normal/homogeneous significant in normality test/homogeneity test;

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

Table 5. Statistical analysis results of Post Hoc Test / Multiple comparison / Tukey HSD TNF- α on day 7
Between Groups

Group		Post Hoc Test / Tukey HSD (p)* Day-7
Control	DBBM	0,125
	FDBB	0,111
	dc-FDBB	0,005*
DBMM	FDBB	1,000
	dc-FDBB	0,390
FDBB	dc-FDBB	0,427

*) p-value <0.05 there is a significant difference in post hoc multiple comparison between groups on day 7

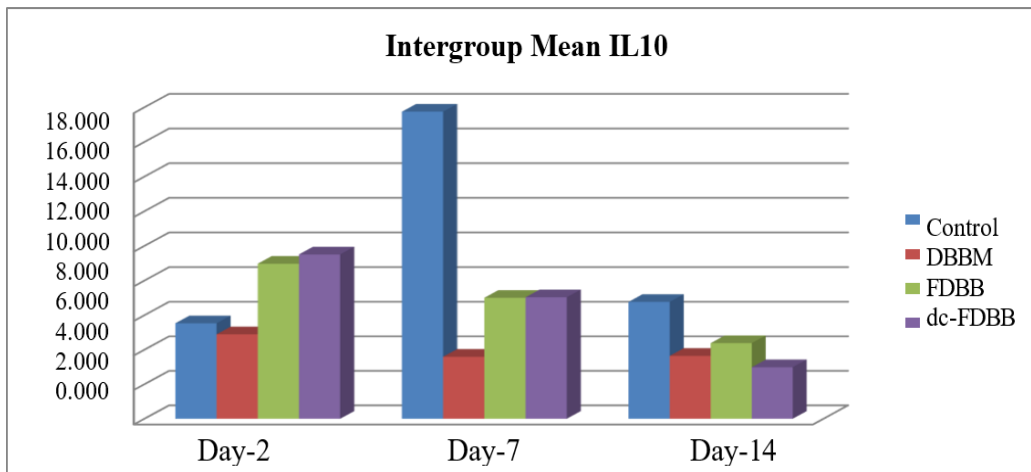


Figure 5. Graph of mean serum IL-10 levels

Table 6. Description of IL-10 data; mean & SD (pg/ml) between groups and between observation times

Group	Days 2		Days 7		Days 14	
	Mean	SD	Mean	Mean	SD	Mean
Control	5,532	3,318	17,748	23,168	6,769	2,844
DBBM	4,892	3,424	3,593	2,464	3,645	2,563
FDBB	8,971	12,239	7,005	1,761	4,383	4,678
dc-FDBB	9,506	6,122	7,036	5,676	2,986	1,983

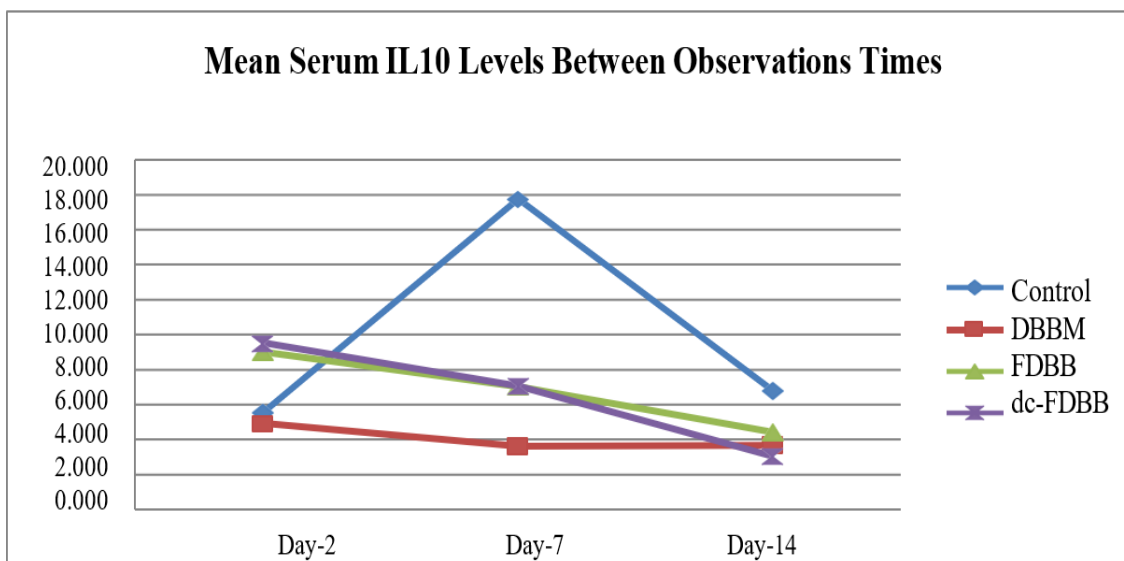


Figure 6. Graph of Mean Serum IL-10 Levels between groups and between observation times there are differences between groups on days 2, 7, 14

Table 7. Results of statistical analysis of IL-10 data (normality test, Levene Test homogeneity test, and Kruskal-Wallis test) between observation times on days 2, 7, 14

Days	Group	Mean ± SD (pg/ml)	Saphiro-wilk (p)*	Lavene's test (p)*	One Way Anova (p)**	Kruskal-Wallis (p)**
Day 2	Control	5,532 ± 3,318	0,427*	-		0,743
	DBBM	4,892 ± 3,424	0,679*			
	FDBB	8,971 ± 12,239	0,040			
	dc-FDBB	9,506 ± 6,122	0,716*			
Day 7	Control	17,748 ± 23,168	0,364*	0,160*	0,349	-
	DBBM	3,593 ± 2,464	0,216*			
	FDBB	7,005 ± 1,761	0,652*			
	dc-FDBB	7,036 ± 5,676	0,273*			
Day 14	Control	6,769 ± 2,844	0,359*	0,098*	0,374	-
	DBBM	3,645 ± 2,563	0,575*			
	FDBB	4,383 ± 4,678	0,237*			
	dc-FDBB	2,986 ± 1,983	0,446*			

*) *p-value > 0.05 normal/homogeneous significant in normality test/homogeneity test;*

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

DISCUSSION

Scaffold DBBM was chosen as the positive control group due to its widespread use and biocompatible nature. However, DBBM has limitations as it only contains inorganic components, leading to decreased osteoinductive properties and very slow degradation, resulting in impaired healing with graft particles integrating into new bone growth⁷. Additionally, this study included a negative control group consisting of rats undergoing the same surgical procedure without implantation to distinguish between cytokine levels measurable as a response to surgery and as a response to biomaterial implantation.

In vivo research was conducted to examine the body's response following the implantation of a scaffold. Subcutaneous implantation of a scaffold in Wistar strain *Rattus norvegicus* was chosen for Subcutaneous implantation of a scaffold in Wistar strain *Rattus norvegicus* was undertaken for research involving small animals serving as preclinical testing models assessing immune reactivity and revascularization capacity allowing assessment of tissue compatibility such as potential sensitization irritation and systemic toxicity.¹²

In this study, serum levels of pro-inflammatory cytokines TNF-α and IL-6 were measured to assess inflammatory activity, while the anti-inflammatory cytokine IL-10 was measured to assess anti-inflammatory activity following scaffold implantation on days 2, 7, and 14. Systemic cytokine profiles can serve as additional parameters for evaluating the in vivo response to new biomaterials or modifications, as well as for monitoring or predicting outcomes post-implantation. Analyzing different populations of

inflammatory cells at different time points can help clarify the reasons for observed differences in cytokine kinetics over time and their correlations with each other, as well as potential associations with serum cytokine levels and local reactions at the implantation site. This can also aid in understanding the relationship between material properties, inflammatory responses, systemic and local immune-related material reactions, and implant function, thus elucidating the role of cytokine mediation in these interactions.¹⁸ In this study, days 2, 7, and 14 were chosen to capture both acute and chronic inflammatory responses.

Scaffold DBBM was chosen as the positive control group due to its widespread use and biocompatible nature. However, DBBM has limitations as it only contains inorganic components, leading to decreased osteoinductive properties and very slow degradation, resulting in impaired healing with graft particles integrating into new bone growth.⁷ Additionally, this study included a negative control group consisting of rats undergoing the same surgical procedure without implantation to distinguish between cytokine levels measurable as a response to surgery and as a response to biomaterial implantation.

The results of this study indicate that TNF-α levels showed the highest mean on days 2, 7, and 14 in the same order of groups, namely the dc-FDBB group, followed by the FDBB group, DBBM group, and lowest in the control group (Figure 3). The pattern of TNF-α levels in the graphs for the FDBB, dc-FDBB, and DBBM groups showed a similar pattern, with the highest levels on day 2, followed by a decrease on days 7 and 14. In contrast, the control group displayed a different pattern, with levels decreasing from day 2 to day 7, and slightly increasing by day 14. The decrease in levels from day 2 to day 7, which

was the lowest point, indicates that in the control group there was no biomaterial present, allowing the inflammation process to be resolved for initial wound healing, with wound healing at the incision site completed by day 7. The differences observed in the scaffold groups are due to the body's response to a foreign object, resulting in a more gradual decrease in TNF- α levels on the graph. However, the FDBB, dc-FDBB, and DBBM groups displayed a consistent pattern of decreasing TNF- α levels until day 14, indicating resolution of inflammation and that the presence of biomaterial did not lead to prolonged inflammation.

The highest TNF- α levels were found in the dc-FDBB group on day 2 post-implantation, indicating that the decellularized scaffold still triggered a fairly strong acute inflammatory response. This could be due to the presence of antigenic residues or a decellularization process that had not completely eliminated cellular components and immunogenic proteins. On the other hand, the DBBM group showed relatively lower TNF- α levels, reflecting the immunological stability of synthetic materials that have been widely used clinically.

The significant decrease in TNF- α levels on day 14 across all groups indicates that the acute inflammatory response was temporary and did not develop into chronic inflammation. Biologically, this signifies that the immune system successfully resolved the inflammatory phase and began entering the resolution phase, which is important in the tissue healing process and scaffold integration.

The highest levels of TNF- α on day 2 align with the theory that TNF- α is a proinflammatory cytokine that appears in the early stages of inflammation. The release of TNF- α increases 24 hours after implantation of bovine bone scaffold and decreases after 72 hours. TNF- α plays a role in the synthesis of inflammatory mediator prostaglandin, is involved in bone resorption, and triggers the synthesis of proteases through osteoblast and fibroblast as well as impacts the osteoprotegerin (OPG) / RANKL system.¹⁵ This corresponds with Paschalia and Antonios who state that TNF- α concentrations in experimental animals peak 24 hours after bone injury and return to normal levels after 72 hours.¹⁵

The DBBM, FDBB, and dc FDBB groups exhibit similar patterns, but with higher levels compared to the control group, indicating an increase in TNF- α levels higher than the control group in the presence of biomaterial, suggesting that the increase in TNF- α occurs as a response to the body's reaction to the biomaterial. This is in line with previous studies that have revealed that the theory of biomaterial implantation is always accompanied by injury through surgical procedures.¹⁹ Tissue or organ injury sparks an inflammatory response against biomaterial starting

with formation of temporary matrix rapidly around it. Implantation of hybrid engineered cell materials triggers innate and adaptive immune response against cellular components affecting host response towards material components.¹⁹ However, the DBMM levels are relatively lower because DBMM is composed of inorganic materials only, lacking the proteins that trigger inflammatory responses. On the other hand, FDBB is considered to contain trigger proteins such as the a-gal epitope, leading to a higher level of inflammation. The dc-FDBB, which undergoes a deactivation process during manufacturing to eliminate proteins that trigger inflammatory responses and reduce the release of pro-inflammatory cytokines, actually results in higher levels of TNF- α compared to FDBB. This suggests that the deactivation process is not effective in reducing inflammatory responses. Deactivation process reduces mechanical properties of scaffold significantly due to weakening adhesion strength between organic and inorganic components drastically. A study has reported that mechanical testing emphasizes the important role of organic components, whose quality and morphology directly affect the material's strength and determine its deformation mechanisms.²⁰

In the comparison test with Kruskal Wallis on day 2, a p-value of 0.250 was obtained, showing no difference between groups. On day 7, the One Way ANOVA test indicated a difference between groups with a p-value of 0.009, and the Post Hoc test with Tukey HSD showed a difference between the control group and dc-FDBB group with a p-value of 0.005. However, on day 14, the One Way ANOVA test with a p-value of 0.324 revealed no difference between groups. The disparity between the dc-FDBB and control groups on day 7 is attributed to a gradual decrease in TNF- α levels in the dc-FDBB group due to the presence of dc-FDBB biomaterial, resulting in wound healing by day 7. Overall, TNF- α levels in the FDBB and dc-FDBB groups approached the gold standard, DBBM, with no significant differences observed across groups. Both FDBB and dc-FDBB are well tolerated by the body, as they do not elicit excessive inflammatory responses and follow a pattern of decline, preventing prolonged inflammation.

The IL-6 levels showed the highest mean on day 2 in the DBBM group, on day 7 in the dc-FDBB group, and on day 14 in the control group. The graphical pattern displays a similar trend in the FDBB, dc-FDBB, and control groups, with an increase in the mean value on day 7 compared to day 2, and a decrease on day 14 compared to day 7. The graphical pattern indicates a different trend for the DBBM group, which consistently decreases from the beginning of implantation to day 14 (Figure 2).

Statistical analysis utilizing One Way ANOVA with p values on day 2, 7, and 14 of $p = 0.530$, $p = 0.279$, and $p = 0.790$ respectively, indicates that there is no significant difference among the four groups on days 2,

7, and 14. These findings align with the research conducted by You et al, which observed an increase in IL-6 levels on day 7, as IL-6 synthesis was stimulated by IL-1 and TNF- α especially in the initial stages of inflammation, resulting in elevated IL-6 levels specifically on day 7.⁹ Subsequently, IL-6 inhibits the activity of IL-1 and TNF- α , leading to a decrease in pro-inflammatory cytokines and facilitating tissue regeneration and repair.⁹ The decrease in IL-6 levels as depicted in Figure 5.1 on day 14 signifies a dynamic reduction in serum pro-inflammatory IL-6 levels post subcutaneous implantation, thereby preventing prolonged inflammation.

IL-6, as a systemic proinflammatory cytokine, showed different fluctuation patterns over time depending on the scaffold type. In the dc-FDBB group, IL-6 levels peaked on day 7, indicating that the systemic inflammatory response lasted longer compared to other groups. This may be due to the ongoing interaction between the immune system and scaffold components that have not been fully recognized as "self." The delayed IL-6 peak also suggests the possibility of a secondary immune reaction to ECM degradation.

Meanwhile, in the DBBM and FDBB groups, IL-6 levels were relatively lower and decreased steadily, indicating that these scaffolds adapted more quickly in host tissues. These findings reinforce the hypothesis that biological scaffolds, especially non-decellularized ones, have natural immunomodulatory capabilities, although their long-term effects still need to be assessed.

During the initial phase of scaffold implantation, the body responds to it as a foreign object, leading to an inflammatory reaction. In the early stages of inflammation, macrophages (M1) release pro-inflammatory cytokines such as IL-6 and TNF- α , and act as Antigen Presenting Cells (APCs) presenting MHC class II to Naïve CD4+ cells, resulting in Th1 producing cytokines IFN- γ , IL-2, TNF- α that also play a role in inflammation. This is what causes an increase in TNF- α levels on day 2 and a decrease starting on day 3, as indicated by the findings of this study, in order to transition to adaptive inflammatory response and tissue repair.¹⁵

This research findings are further supported by a preliminary in vitro study demonstrating that FDBX granules do not elicit prolonged immune responses.²¹ The immune response to scaffolds is directly dependent on the amount of residual DNA, as nucleic acid is recognized by host immune cells. While in vitro research has also proven that both FDBB and dc-FDBB models do not contain significant amounts of antigenic residues.⁸

The results of the serum IL 10 levels described in Table 6, Figure 5, and Graph 6 illustrate a consistent

pattern of IL 10 levels in both the FDBB and dc-FDBB groups, peaking on day 2 before decreasing on day 7 in both groups, and on day 14 in the FDBB and dc-FDBB groups. The elevated levels on day 2 indicate a body response in releasing anti-inflammatory cytokines as part of the M2 response to reduce inflammation. In the control group, the mean IL 10 levels increased on day 7 and decreased on day 14, aligning with the findings of You et al who observed an increase in IL-10 levels on days 7, 14, and 28 before a subsequent decrease.⁹

The improvement on the early morning of the day 2 compared to the control group indicates an anti-inflammatory response in the FDBB and dc FDBB groups, which is beneficial in reducing inflammation. This phenomenon was not observed in the DBBM group, as evidenced by the relatively lowest and consistent levels of IL 10 on days 2, 7, and 14.

IL-10 was chosen as the anti-inflammatory indicator in this study due to its status as a multifunctional cytokine that acts as a critical immunomodulator, playing a positive role in maintaining immune balance, attenuating acute inflammatory feedback, and generating a negative feedback loop that diminishes the release of inflammatory mediators.¹⁶ IL-10 reportedly inhibits cytokine synthesis pretty effectively and deactivates macrophages thereby preventing deleterious effects of excessive macrophage activation during various inflammatory responses.⁹

The IL10 levels in the low-DBBM group may be attributed to the fact that DBBM contains only inorganic materials, whereas they are higher in the FDBB and dc FDBB groups due to the presence of organic materials, ECM, and growth factors that can stimulate towards M2 response and anti-inflammatory cytokine IL 10 production. When acute inflammation by M1 is resolved without prolonged or chronic inflammation, M1 will polarize into M2 and produce IL-10, which plays a role in down-regulating the immune response and stimulating growth factors (VEGF, FGF, PDGF) in the ECM, thus triggering angiogenesis and proliferation as a tissue regeneration process towards healing. The M2 response occurs when the scaffold is properly decellularized or xenoantigens are eliminated in the initial M1 response, leading to down-regulation of the immune response.²²

IL-10, as an anti-inflammatory cytokine, experienced a significant initial increase on day 2, particularly in the FDBB and dc-FDBB groups. This phenomenon indicates that despite early inflammation, the body also immediately triggers compensatory mechanisms to suppress the inflammatory response through IL-10 secretion. This suggests the potential of biological scaffolds to promote a shift from the inflammatory phase to the regenerative phase.

Specifically in dc-FDBB, IL-10 levels remained high until day 7, which may reflect the immune system's efforts to balance the proinflammatory response due to remaining antigenic residues. Meanwhile, in DBBM, the

increase in IL-10 was not as pronounced, reflecting that synthetic scaffolds are more immunologically neutral and do not strongly stimulate the body's adaptive response.

The macrophage response toward M2 occurs during the implantation of FDBB and dcFDBB scaffolds, where materials believed to trigger inflammation like the protein a-gal epitope can be eliminated in the early M1 response on days 1 and 2, leading to a shift from an M1 response towards an M2 response through T regulatory or T suppressor cells. M2 produces pro anti-inflammatory cytokines such as IL-10, IL-4, IL-13, TGF- β , MMPs, as well as Growth Factors (VEGF, FGF, PDGF). IL-10, as an anti-inflammatory cytokine, plays a role in down-regulating the immune response.²² IL-10 acts as an immune response in the late stage of inflammation by inhibiting the synthesis of pro-inflammatory cytokines IL-1, IL-6, and TNF- α , and deactivating macrophages as a role in down-regulating the immune system. Additionally, IL-10 activates B cells to form antibodies as a transition to the remodeling phase, and also stimulates growth factors (VEGF, FGF, PDGF) present in the ECM, which play a role in angiogenesis and proliferation during the remodeling and healing phases.²³

Ultimately, the results of the Kruskal-Wallis statistical test on day 2 indicate $p = 0.743$. The One Way ANOVA test results on days 7 and 14 with $p = 0.349$ and 0.374 respectively, show that there is no significant difference in serum IL 10 levels post-implantation of decellularized and non-decellularized scaffolds across all groups. The design of biomaterials has evolved beyond simply developing passive materials that limit immune responses. It now involves enhancing tissue repair through the ability to down-regulate unwanted inflammatory responses. The relationship between immune response and tissue repair is highly complex, and the current challenge is to develop biomaterials capable of modulating the immune system to stimulate tissue and organ repair.²⁴ Around 4 days post inflammation Treg cells arrive at inflamed site and modulate immune response by heavily promoting shift from M1 macrophage subtype to anti-inflammatory M2 subtype thereby enabling stem cell proliferation and differentiation.²⁵ This aligns with the findings of this study that on day 7 post-implantation of FDBB and dc FDBB, serum levels of the anti-inflammatory cytokine IL-10 increased as a result of the M2 response.

FDBB and DC FDBB offer advantages over DBBM because these materials retain Extracellular Matrix comprised of various tissue-specific macromolecules like collagen and elastin naturally. Decellularized scaffold alters plasticity of adherent macrophages thereby substantially enhancing inflammation and profoundly modulating immune regulation quite effectively. Circulating macrophages induce

necessary reactions pretty quickly when activated by heterogeneous extracellular matrix leading to anti-inflammatory responses or constructive remodeling.²⁶

CONCLUSION

The findings of this study demonstrate a decrease in the levels of serum pro-inflammatory cytokines (IL-6 and TNF α), indicating a shift from pro-inflammatory cytokine levels towards improvement (M2 response) rather than prolonged inflammation (M1 response) in the freeze-dried bovine bone scaffold decellularization group. Of particular interest is the increase in IL-10 levels following the implantation of dcFDBB and FDBB compared to DBBM, suggesting that FDBB and dcFDBB may possess the potential for immunomodulation due to their ECM and growth factor properties. The results of the comparative test indicate no significant difference in the levels of pro-inflammatory cytokines IL 6, TNF α in the serum post-implantation between desellularized and non-desellularized bovine bone scaffolds when compared to the control group. This suggests that both scaffolds do not elicit prolonged immune response (M1 response) and instead promote resolution (M2 response). These findings are consistent with previous research that found no significant differences in inflammatory cytokines TNF- α , IL-6, and ALP levels between the control group and those implanted with xenogeneic materials.⁶

Overall, these results indicate that the choice of scaffold type significantly influences the dynamics of the inflammatory response in vivo. Biological scaffolds have advantages in triggering anti-inflammatory and regenerative responses, but also risk causing higher initial inflammation if the decellularization process is not optimal. Conversely, synthetic scaffolds such as DBBM tend to be more immunologically stable but have limitations in supporting active regenerative responses. Thus, a balance is needed between decellularization effectiveness, the scaffold's ability to support regeneration, and minimal inflammatory triggers. The results of this study can serve as a foundation for developing biomaterials with more precise bioengineering approaches, and are important in planning clinical applications of scaffolds in bone tissue engineering or other soft tissues.\

DECLARATIONS

Ethics approval and consent to participate

Ethical approval was obtained from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Reference 2.KEH.125.09.2022).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Funding

This research received no external funding.

REFERENCES

- Oliveira MTF, Rocha FS, Batista JD, de Moraes SLC, Zanetta-Barbosa D. *Reconstruction of Mandibular Defects. In A Textbook of Advanced Oral and Maxillofacial Surgery. A Textbook of Advanced Oral and Maxillofacial Surgery. Mohammad Hosein Kalantar Motamedi.* IntechOpen; 2013.
- Wang W, Yeung KWK. Bone grafts and biomaterials substitutes for bone defect repair: A review. *Bioact Mater.* 2017;2(4):224-247.
- Tang G, Liu Z, Liu Y, et al. Recent trends in the development of bone regenerative biomaterials. *Front Cell Dev Biol.* 2021;9:665813.
- Ghassemi T, Shahroodi A, Ebrahimzadeh MH, Mousavian A, Movaffagh J, Moradi A. Current concepts in scaffolding for bone tissue engineering. *Arch bone Jt Surg.* 2018;6(2):90.
- Wong ML, Griffiths LG. Immunogenicity in xenogeneic scaffold generation: antigen removal vs. decellularization. *Acta Biomater.* 2014;10(5):1806-1816.
- Sun X, Liu C, Shi Y, et al. The assessment of xenogeneic bone immunotoxicity and risk management study. *Biomed Eng Online.* 2019;18:1-14.
- Kamadajaja DB, Satriyo H, Setyawan A, et al. Analyses of bone regeneration capacity of freeze-dried bovine bone and combined deproteinized-demineralized bovine bone particles in mandibular defects: The potential application of biological forms of bovine-bone filler. *Eur J Dent.* 2022;16(02):403-413.
- Montessory M, Kamadajaja DB, Sumarta NPM, Rizqiawan A, Rahman MZ. Freeze-Dried Bovine Bone as Xenogenic Scaffold: Does Decellularization Lower Its Antigenic Potential? *J Int Dent Med Res.* Published online 2022.
- You L, Weikang X, Lifeng Y, et al. In vivo immunogenicity of bovine bone removed by a novel decellularization protocol based on supercritical carbon dioxide. *Artif cells, nanomedicine, Biotechnol.* 2018;46(sup2):334-344.
- Ahmed AF, Abdulkareem MM. Essentials of Pre- and Post-Operative Evaluation of Total Hip Arthroplasty. *Pharmacol Med REPORTS, Orthop Illn DETAILS.* 2024;3(3):84-100. doi:10.55047/comorbid.v3i3.1340
- Massaro MS, Palek R, Rosendorf J, Červenková L, Liška V, Moulisova V. Decellularized xenogeneic scaffolds in transplantation and tissue engineering: Immunogenicity versus positive cell stimulation. *Mater Sci Eng C.* 2021;127:112203.
- Khorrampirou R, Go JL, Noble C, et al. A novel surgical technique for a rat subcutaneous implantation of a tissue engineered scaffold. *Acta Histochem.* 2018;120(3):282-291.
- Baseri M, Radmand F, Hamed R, Yousefi M, Kafil HS. Immunological aspects of dental implant rejection. *Biomed Res Int.* 2020;2020(1):7279509.
- Choy E, Rose-John S. Interleukin-6 as a multifunctional regulator: inflammation, immune response, and fibrosis. *J Scleroderma Relat Disord.* 2017;2(2_suppl):S1-S5.
- Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue Eng Part B Rev.* 2008;14(2):179-186.
- Male D. *Immunology: An Illustrated Outline.* CRC Press; 2021.
- Lemeshow S, Hosmer DW, Klar J, Lwanga SK. Besar sampel dalam penelitian kesehatan. *Yogyakarta Gajah Mada Univ.* Published online 1997.
- Walschus U, Hoene A, Patrzyk M, et al. Serum profile of pro-and anti-inflammatory cytokines in rats following implantation of low-temperature plasma-modified titanium plates. *J Mater Sci Mater Med.* 2012;23:1299-1307.
- Franz S, Rammelt S, Scharnweber D, Simon JC. Immune responses to implants—a review of the implications for the design of immunomodulatory biomaterials. *Biomaterials.* 2011;32(28):6692-6709.
- Anisimova NY, Kiselevsky M V, Sukhorukova I V, Shvindina N V, Shtansky D V. Fabrication method, structure, mechanical, and biological properties of decellularized extracellular matrix for replacement of wide bone tissue defects. *J Mech Behav Biomed Mater.* 2015;49:255-268. doi:10.1016/j.jmbbm.2015.05.009
- Humidat AKM, Kamadajaja DB, Bianto C, Rasyida AZ, Harijadi A. Effect of freeze-dried bovine bone xenograft on tumor necrosis factor-alpha secretion in human peripheral blood mononuclear cells. *Asian Jr Microbiol Biotech Env Sc.* 2018;20:S88-S92.
- Lebaudy E, Fournel S, Lavallo P, Vrana NE, Gribova V. Recent advances in antiinflammatory material design. *Adv Healthc Mater.* 2021;10(1):2001373.
- Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev.* 2008;226(1):205-218.
- Julier Z, Park AJ, Briquez PS, Martino MM. Promoting tissue regeneration by modulating the immune system. *Acta Biomater.* 2017;53:13-28.
- Crupi A, Costa A, Tarnok A, Melzer S, Teodori L. Inflammation in tissue engineering: The Janus between engraftment and rejection. *Eur J Immunol.* 2015;45(12):3222-3236.
- Li X, Xie H. Decellularized materials, Preparations and Biomedical Applications. *1st ed Singapore Springer.* Published online 2021.