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# Immunogenicity Response Test of Human Peripheral Blood Mononuclear Cells Culture After Application of Scaffold Freeze-Dried Bovine Bone, Decellularized Freeze-Dried Bovine Bone, and Deproteinized Bovine Bone Materials (In Vitro Laboratory Observational Research)

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**ABSTRACT**

Bovine bone xenograft's physical and chemical structure is similar to human bone; therefore, it has been a choice of bone graft material other than autograft. Freeze-dried bovine bone (FDBB) scaffolds with or without decellularization still have organic content and will react to the body's immunity and trigger an inflammatory response. At the same time, Deproteinized Bovine Bone Material (DBBM) has been widely used because it is not antigenic. Peripheral Blood Mononuclear Cell culture was used to prove the inflammatory response through cytokine secretion levels of Interleukin-1 and Interleukin-10. This research aims to evaluate and compare IL-1 and IL-10 secretion levels as inflammatory markers in FDBB, dc-FDBB and DBBM scaffolds. The method used in this research are FDBB, dc-FDBB, and DBBM scaffolds were observed in human Peripheral Blood Mononuclear Cells conditioned medium for 1, 3, and 7 days to see the comparison of IL-1 and IL-10 secretion levels by ELISA using an ELISA Reader at 450 nm absorbent with a colour change to intense yellow. In addition, observations of Optical Density results were made on the three scaffolds. Furthermore, a data distribution test was conducted with Shapiro-Wilk and data analysis with ANOVA. The results in the observation of Interleukin-1 cytokine secretion levels show that FDBB and DBBM scaffolds have decreased secretion levels from day 1, 3 to 7, but dc-FDBB scaffolds appear to have increased Interleukin 1 secretion levels on day three, but decreased on day 7. In the observation of IL-10 secretion, both FDBB, dc-FDBB, and DBBM scaffolds experienced a significant decrease in secretion levels compared to the control group. There was no significant difference between the FDBB, dc-FDBB and DBBM groups on days 1, 3 and 7 of observation ($p>0.05$). The observations showed that the three scaffolds were not antigenic.

***Keywords:*** *Scaffold, Cytokine, Interleukin, Secretion, Immunity*

## 1. INTRODUCTION

Tooth extraction will be followed by alveolar bone resorption which begins rapidly and continues for years [1]. Bone development in embryos happens through two distinct processes which are intramembranous ossification and endochondral ossification. Intramembranous ossification starts with the accumulation of mesenchymal cells that transform directly into bone cells. Conversely, endochondral ossification is a long process characterized by bone development through cartilage intermediates [2]. Bone tissue has the ability to undergo structural changes by engaging in two main processes which are modeling and remodeling. Remodeling specifically involves the removal and replacement of old bone tissue with new bone, carried out by Bone Multicellular Units (BMU) in a well-coordinated and regulated manner with the participation of osteocytes, osteoblasts, and osteoclasts [3].

Bone graft involves replacing missing bone using materials from the patient's body, artificial substitutes, or natural materials. This procedure is performed because bone tissue can fully regenerate when given the necessary room to grow [4]. As new bone forms, it typically replaces the graft material entirely, ultimately leading to the integration of a new bone area [5].

Bone graft materials are divided into 4 major groups: autograft, allograft, xenograft, and synthetic graft [6]. Autogenous grafts are harvested from an individual's own body, whereas allografts are sourced from one person and transplanted into another person of the same species with different genetic makeup [7]. The disadvantages of autograft are patient morbidity due to required surgery, extended operation time, increased risk of infection and reduced bone strength in the harvested area, while the disadvantages of allograft are limited donor quantity, risk of disease transmission, and relatively more expensive prices. Therefore, alternative bone graft materials are needed to overcome the disadvantages of autograft and allograft [8].

Numerous research have been carried out in search of substitutes for autografts and allografts, with one of the options being the utilization of bovine xenografts - bone grafts sourced from bovine bone. These have advantages such as being abundant in supply, having a composition resembling human bone, and being cost-effective. However, a drawback is the potential for antigenic reactions leading to heightened inflammation when using this type of bone graft [9].

One commonly used type of bovine xenograft is freeze-dried bovine bone, which undergoes freezing and drying procedures to preserve its physical and chemical integrity while minimizing antigenic responses. Studies have demonstrated that this xenograft is non-toxic and can effectively promote the proliferation and differentiation of fibroblast cells in rat cultures [10].

The application of bone graft to alveolar bone defects will trigger the body's immune response. The peripheral mononuclear blood cell system consists of blood leukocytes and tissue cells derived from leukocytes in preventing various diseases. Th2 lymphocytes can produce IL-4, IL-5, IL-6, and IL-10 cytokines that do not activate macrophages so that graft integration and remodeling can proceed well [11]. After alveolar bone grafting, bone healing occurs through an inflammatory process, leading to the growth and specialization of bone marrow mesenchymal stem cells that generate fresh bone tissue. Bone marrow mesenchymal stem cell tissue regeneration can originate from the cancellous itself and from periosteal tissue [12]. Inflammatory indicators that are often studied are monocytes, lymphocytes, macrophages, and inflammatory mediators that circulate in blood or also called Peripheral Blood Mononuclear Cells (PBMC) [13].

ELISA (Enzyme Linked Immunosorbent Assay) operates on the fundamental principle that antigens specifically bind to their corresponding antibodies, enabling the detection of minute quantities of biological molecules like proteins, peptides, hormones, or antibodies in liquid specimens. The technique employs enzyme-tagged antigens and antibodies as detection tools. The process involves fixing antigens from liquid samples onto solid surfaces (typically 96-well microplates), where they capture specific antibodies. These bound antibodies are subsequently identified using secondary enzymes that generate color changes or fluorescent signals, confirming antigen presence. The intensity of these colorimetric or fluorescent reactions allows for both qualitative identification and quantitative measurement of target molecules. Using fluorogenic substrates enhances detection sensitivity and provides more precise determination of antigen concentrations in test samples [14].

Based on the above description, researchers are interested in conducting research on immunogenicity responses in Peripheral Blood Mononuclear Cells culture after administration of Freeze-Dried Bovine Bone, dc-Freeze Dried Bovine Bone, and Deproteinized Bovine Bone Material scaffolds for 1, 3 and 7 days. Research on this matter has been conducted previously on experimental animals. Researchers want to continue this research using human blood culture (Human Peripheral Blood Mononuclear Cells). This research is conducted by taking blood samples from humans with the same blood type. Blood samples are taken from subjects who have uniformity in terms of gender, blood type, age, and dietary patterns since the immune condition of the subject can be influenced by the subject's dietary patterns.

## 2. RESEARCH METHODS

### Research Type

This study employs a post-test only control group design by assessing the secretion levels of IL-1 and IL-10 in hPBMC cultures after exposure to FDBB, dc-FDBB, and DBBM conditioned media at a concentration of 2.5% for 1, 3, and 7 days.

### Research Sample

This study utilized scaffold preparations of FDBB, dc-FDBB, and DBBM, along with hPBMC cultures derived from healthy volunteers (male PPDGS participants, blood type O, with no comorbidities). All donors were screened to be disease-free, with uniformity in sex and blood type to ensure sample homogeneity.

a) Group I: Observation of IL-1 and IL-10 secretion in 5 samples of FDBB, dc-FDBB, and DBBM in hPBMCs culture after adding 2.5% conditioned medium as treatment group on day 1
b) Group II: Observation of IL-1 and IL-10 secretion in 5 samples of FDBB, dc-FDBB, and DBBM in hPBMCs culture after adding 2.5% conditioned medium as treatment group on day 3

c) Group III: Observation of IL-1 and IL-10 secretion in 5 samples of FDBB, dc-FDBB, and DBBM in hPBMCs culture after adding 2.5% conditioned medium as treatment group on day 7
d) Group IV: Observation of IL-1 and IL-10 secretion in 5 samples of hPBMCs culture without conditioned medium as control group without treatment for days 1, 3 and 7

**Sample Size Calculation (Federer Formula)**
The sample size used in this research is calculated based on Federer calculation formula because there is no preliminary research before, with the following formula:

$$(\boldsymbol{n}-\mathbf{1})(\boldsymbol{t}-\mathbf{1}) \geq \mathbf{15}\ (n-1)$$
$$(5-1) \geq 15$$
$$n \geq 5$$

Where:
n = number of replications
t = number of treatments

The above minimum sample number calculation formula can be performed because there is no preliminary research. The number of treatments in this research is 3 treatments (days 1, 3, 7) so the number of replications (n) obtained is 30 samples for treatment and control groups. Considering the possibility of data homogeneity to be obtained, researchers use 5 samples each for each treatment group and control group. The total number of samples used is 30 samples.

**Research Grouping**
Research samples are divided into 2 groups: control group and treatment group.

**Control Group**
Observation of IL-1 and IL-10 secretion in hPBMCs culture on days 1, 3, and 7.

**Treatment Group**
Observation of IL-1 and IL-10 secretion in hPBMCs culture on days 1, 3, 7 after application of FDBB, dc-FDBB, and DBBM 2.5% conditioned medium.

**Research Variables**
Research variables in this study consist of several types of variables that have different roles. Independent variables include FDBB, dc-FDBB, and DBBM applied to hPBMCs culture. Control variables consist of several important aspects, namely tools and materials used during research, medium used for cell culture, and micro environment around cell culture that can affect experimental results. While the dependent variable in this research is IL-1 and IL-10 secretion in hPBMCs culture, which becomes an indicator of cell response to treatment with independent variables.

**Operational Definition**
a) Human Peripheral Blood Mononuclear Cells: Peripheral blood from volunteer donors 5-10 ml and placed in blood container (EDTA tube) and cultured according to existing protocols.
b) Freeze Dried Bovine Bone (FDBB): FDBB is bone graft material made from cortical and cancellous bone pieces from bovine femur region with size 5 x 3 x 3 mm that has undergone freezing and drying processes, produced by National Research and Innovation Agency (BRIN), Jakarta.
c) Scaffold Decellularized freeze dried bovine cancelous bone (dc-FDBB): Scaffold material made from bovine cancellous bone with freeze drying process by soaking using ionic sodium duodecyl sulfate (SDS) material at National Research and Innovation Agency, Jakarta with size 5 x 5 x 3 mm.
d) Scaffold Deproteinized bovine cancelous bone matrix (DBBM): Scaffold material made from bovine cancellous bone with decellularization and deproteinization process through burning or furnace process at 1000°C at National Research and Innovation Agency, Jakarta with size 5x5x3 mm.
e) IL-1 and IL-10 Secretion: IL-1 and IL-10 secretion is the color that appears in the cell cytoplasmic membrane using Enzyme Linked Immunosorbent Assay (ELISA) technique after being given substrate solution F. The reaction is stopped by adding 100 ml stop solution to each well. The solution color changes from blue to yellow. Absorbance is read at 450nm wavelength within 30 minutes.

**Research Materials and Instruments**
Materials used in this research include 70% Alcohol, distilled water, 100µL blue tip, EDTA, ficoll Isopaque gradient 1.077 g/ml, disposable filter, foetal calf serum (FCS), fungizone, cell growth medium (alpha medium), Rosewell Park Memorial

Institutes medium (RPMI), methylin blue, Penicillin 100IU, Petridish 5 cm and 10 cm, phosphate buffer saline (PBS), 15 ml and 50 ml disposable tubes, trypsin 0.25%, 0-20 µL yellow tip.

Equipment used consists of Autoclave, 300 ml and 500 ml backer glass, ELISA Reader, 150 ml and 250 ml Erlenmeyer, Medium filter, Freezer -20°C and -80°C, 5 ml, 10 ml, and 20 ml glass pipettes, CO2 incubator, drying incubator, 0-200 µL and 1000 µL micropipette, inverted microscope, automatic pipette, 4°C refrigerator, sterile room equipped with UV, adjustable refrigerator centrifuge, CO2 tank, CO2 regulator.

**Research Procedure**

The research is divided into 2 research groups: control group and treatment group. In the treatment group, observations are made on days 1, 3, and 7. Based on the sample number calculation formula, this research obtained 5 samples in each control group and treatment group, so the total number of research samples used is 25 samples. After sample grouping, IL-1 and IL-10 secretion observations are performed using ELISA Reader.

**Research Scheme**

This research is conducted to analyze the number of cells in hPBMSc culture that secrete IL-1 and IL-10 in the control group (without FDBBX Conditioned medium 2.5% administration) and the group after FDBBX conditioned medium 2.5% administration in in-vitro culture medium on days 1, 3, 7 observation. IL-1 and IL-10 secretion measurement in samples using Enzyme-linked Immunosorbent Assay (ELISA) reader. Using this instrument for measurement will gather information in the shape of concentration values and optical density, which will indicate the levels of IL-1 and IL-10 secretion.

**Statistical Analysis**

IL-1 and IL-10 secretion level measurement results with ELISA are analyzed statistically with ANOVA test which is first performed normal distribution and homogeneity test.

## 3. RESEARCH RESULTS AND DATA ANALYSIS

In this research, IL-1 and IL-10 secretion level measurement in sample cells using ELISA Reader, where each sample is first given 100 µl/well stopper solution. This measurement technique generates data as Optical Density (OD) values and secretion concentrations. The Optical Density reading represents the intensity of yellow coloration within the cell culture sample. A more intense or deeper yellow color corresponds to a higher Optical Density measurement value. The intensity of yellow color formed is directly proportional to the amount of Interleukin-1 and Interleukin-10 secretion.

In figures 1 and 2 which are bar diagrams, the results of difference test data of mean IL-1 and IL-10 secretion levels between control group and FDBB, dc-FDBB, and DBBM groups in each observation on days 1, 3 and 7 are obtained.

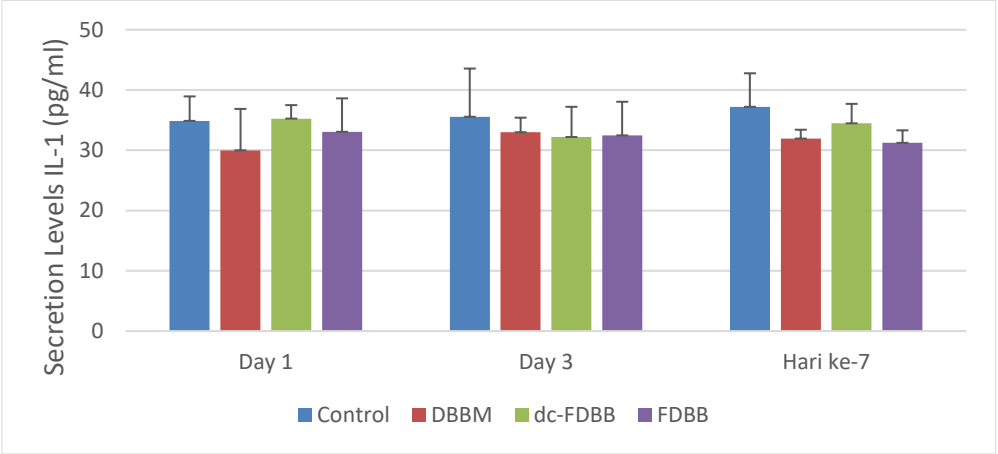


**Figure 1. Results of difference test data of IL-1 secretion levels between control group and FDBB, dc-FDBB, and DBBM groups in each observation on days 1, 3 and 7**

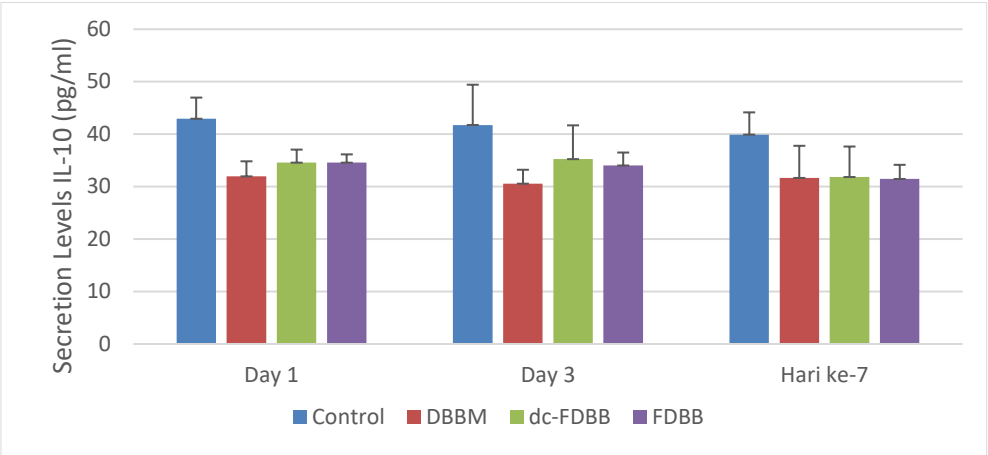


**Figure 2. Results of difference test data of IL-10 secretion levels between control group and FDBB, dc-FDBB, and DBBM groups in each observation on days 1, 3 and 7**

In figure 3, there is no significant difference between IL-1 levels among treatment groups compared to control group (pvalue > 0.05). Figure 4 shows significant difference between IL-10 levels among treatment groups (pvalue < 0.05).

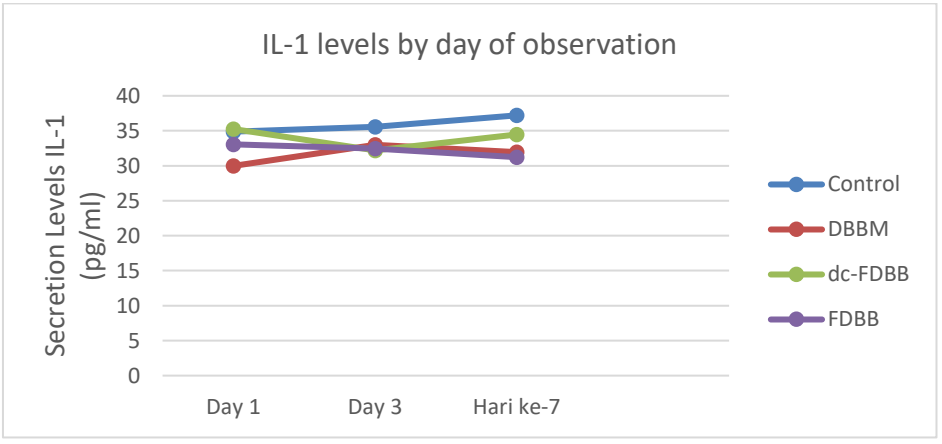


**Figure 3. Evaluation of mean IL-1 secretion levels (pg/ml)**

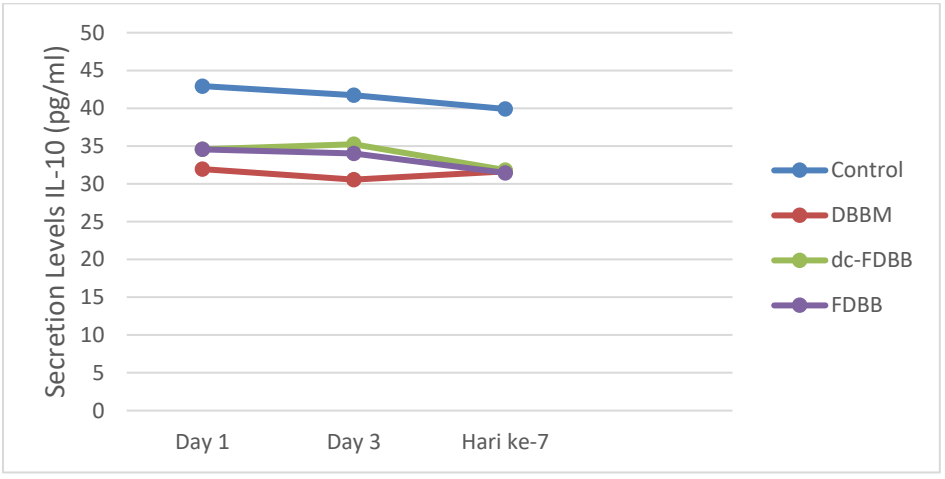


**Figure 4. Evaluation of mean IL-10 secretion levels (pg/ml)**

Before conducting tests and analysis between research groups (Tables 1 and 2), normality tests are performed on each control group and FDBB, dc-FDBB and DBBM groups using Shapiro Wilk test. The obtained data is tested for normality with Shapiro-wilk test to determine data distribution as a requirement for one-way ANOVA difference test. In all research groups, results for treatment groups have values greater than 0.05 ($p \geq 0.05$) which means data in treatment groups are normally distributed.

**Table 1. Normal distribution test results of IL-1 data using Shapiro Wilk Test**

| Group | Observation | N | P value | Description |
|---|---|---|---|---|
| Control | Day 1 | 5 | 0.393 | Normal Distribution |
| | Day 3 | 5 | 0.513 | Normal Distribution |
| | Day 7 | 5 | 0.987 | Normal Distribution |
| DBBM | Day 1 | 5 | 0.071 | Normal Distribution |
| | Day 3 | 5 | 0.401 | Normal Distribution |
| | Day 7 | 5 | 0.120 | Normal Distribution |
| dc-FDBB | Day 1 | 5 | 0.772 | Normal Distribution |
| | Day 3 | 5 | 0.341 | Normal Distribution |
| | Day 7 | 5 | 0.219 | Normal Distribution |
| FDBB | Day 1 | 5 | 0.362 | Normal Distribution |
| | Day 3 | 5 | 0.887 | Normal Distribution |
| | Day 7 | 5 | 0.220 | Normal Distribution |

**Table 2. Normal distribution test results of IL-10 data using Shapiro Wilk Test**

| Group | Observation | N | P value | Description |
|---|---|---|---|---|
| Control | Day 1 | 5 | 0.901 | Normal Distribution |
| | Day 3 | 5 | 0.058 | Normal Distribution |
| | Day 7 | 5 | 0.250 | Normal Distribution |
| DBBM | Day 1 | 5 | 0.580 | Normal Distribution |
| | Day 3 | 5 | 0.474 | Normal Distribution |
| | Day 7 | 5 | 0.387 | Normal Distribution |
| dc-FDBB | Day 1 | 5 | 0.182 | Normal Distribution |
| | Day 3 | 5 | 0.979 | Normal Distribution |
| | Day 7 | 5 | 0.569 | Normal Distribution |
| FDBB | Day 1 | 5 | 0.240 | Normal Distribution |
| | Day 3 | 5 | 0.327 | Normal Distribution |
| | Day 7 | 5 | 0.552 | Normal Distribution |

**Table 3. Normal distribution test results of IL-1 and IL-10 data using Shapiro Wilk Test**

| Shapiro-wilk test | | |
|---|---|---|
| Variable | | p value |
| IL-1 | Optical Density | 0.215 |
| | Secretion Level | 0.160 |
| IL-10 | Optical Density | 0.136 |
| | Secretion Level | 0.058 |

The homogeneity variance test results of control groups show that the variance of the three groups is homogeneous ($p>\alpha$) which means the analysis of variance test requirements are met and the analysis of variance results in the control group show no significant difference in mean IL-1 secretion levels and significant difference in IL-10 between the three observations, therefore further analysis of variance test is needed.

Analysis is continued with homogeneity test on each FDBB, dc-FDBB and DBBM group with control using Levene's Test. Levene's test homogeneity test is run to determine data homogeneity distribution as a requirement for one-way ANOVA difference test to determine data distribution. P value > 0.05 results show that data is homogeneous or comes from homogeneous population.

**Table 4. Normal distribution test results of IL-1 and IL-10 data using Levene Test**

| Levene's Statistic | | |
|---|---|---|
| **Variable** | | **p value** |
| IL-1 | Optical Density | 0.131 |
| | Secretion Level | 0.171 |
| IL-10 | Optical Density | 0.275 |
| | Secretion Level | 0.251 |

To determine differences between treatment groups, difference tests or comparison tests are performed. After meeting normality and homogeneity requirements, data is tested using one-way ANOVA.

**Table 5. Normal distribution test results of IL-1 and IL-10 data using ANOVA Test**

| One-way ANOVA test | | |
|---|---|---|
| **Variable** | | **p value** |
| IL-1 | Optical Density | 0.087 |
| | Secretion Level | 0.059 |
| IL-10 | Optical Density | 0.000 |
| | Secretion Level | 0.000 |

Comparison analysis results show pvalue < 0.05 on IL-10 variable both in terms of Optical density results and secretion levels. Meanwhile, pvalue > 0.05 on IL-1. Thus, it can be concluded that there is a significant difference between IL-10 levels among treatment groups while there is no significant difference in IL-1 examination.

## 4. Discussion

The process of bone repair following trauma involves intricate biological and biomechanical mechanisms that unfold in three distinct stages: the inflammatory stage, the repair stage, and the remodeling stage [15]. The success of bone regeneration relies heavily on the initial inflammatory response, which encompasses both localized and systemic reactions to tissue damage. The healing process involves crucial communication between immune cells and mesenchymal stromal cells (MSC), which work together to regulate bone recovery [15]. Bone grafts serve as substitute materials surgically placed into bone defects to replace missing bone tissue, utilizing materials from the patient's own body, donor sources, natural materials from other species, or synthetic alternatives, with the primary goal of stimulating new bone formation [16].

The primary limitation of xenografts is the occurrence of immune rejection or delayed rejection responses, which can destroy the transplanted material within two weeks. This immune response represents the major obstacle to successful cross-species transplantation. Various processing techniques have been developed for xenografts, including freeze-drying or lyophilization methods, which produce different forms such as freeze-dried bovine bone (FDBB), decellularized FDBB (dc-FDBB), demineralized FDBB (DFDBB), and deproteinized bovine bone mineral (DBBM). DFDBB demonstrates strong bone-inducing properties but lacks bone-conducting abilities due to demineralization effects that compromise structural integrity and accelerate graft absorption. DBBM possesses excellent bone-conducting and mechanical characteristics but lacks bone-inducing capacity because deproteinization eliminates organic components and growth factors. FDBB emerges as the optimal graft material since freeze-drying maintains proteins and growth factors while preserving mechanical strength. However, decellularization of FDBB, while reducing immune rejection risk and creating a natural microenvironment, often diminishes bone-inducing capabilities [17].

Laboratory studies of freeze-dried bovine bone require the preparation of conditioned media at optimal concentrations to ensure effective application of cube or rod particles in hPBMC cultures. Previous research has established that FDBB conditioned medium at 2.5% concentration yields the highest cell survival rates [18]. Cell viability, which measures the proportion of living to dead cells, serves as an indicator of material toxicity [19]. Consequently, this study employs conditioned media from freeze-dried bovine bone, deproteinized bovine bone material, and decellularized freeze-dried bovine bone at 2.5% concentration.

The mechanism of bone healing following alveolar bone grafting involves an inflammatory process followed by bone regeneration through the proliferation and differentiation of bone marrow mesenchymal stem cells, ultimately leading to new bone tissue formation [20]. Bone tissue comprises osteoblasts, osteoclasts, osteocytes, and other fundamental components.

As a dynamic organ, bone maintains equilibrium between formation and resorption processes, involving complex interactions between the immune and skeletal systems. Bone and immune cells not only share the bone marrow environment but also originate from common stem cells and utilize similar regulatory molecules [21]. Immune cells contribute to bone balance regulation, while bone cells influence immune cell growth and development. This bidirectional relationship has led to the term 'osteoimmunology' to describe the interconnected communication between bone and immune systems. Human peripheral blood mononuclear cells (hPBMCs) are blood components with round nuclei, including lymphocytes, monocytes, and macrophages, which play crucial roles in the immune system's defense against infections and foreign substances, making them suitable subjects for studying inflammatory responses [20].

Normal bone remodeling represents a dynamic equilibrium resulting from a sequence of biological processes involving osteoblast and osteoclast cells, which function to remove old damaged bone and create new bone tissue. Osteocytes serve as mechanical sensors and play vital roles in bone remodeling, while bone lining cells initiate the remodeling process through matrix breakdown [21].

Several studies show that the early healing phase is associated with inflammatory processes that can alter normal soft tissue deposits that can change tissue deposition and multinuclear cells on xenograft surfaces where during this time, this phase can be giant cell form. Araujo proposed that neutrophil leukocyte cells (PMN cells) migrate to xenograft particle surfaces during the first healing phase and are replaced with tartrate resistant acid phosphatase (TRAP) positive multinuclear cells (osteoclasts) during the second period [22]. These osteoclasts remove material from xenograft surfaces but disappear from anorganic bovine bone (ABB) granules after 1-2 weeks when followed by osteoblast formation that provides bone material to collagen portions of provisional matrix. Osteoblastic bone formation is usually associated with osteoclastic bone resorption in remodeling processes [23].

Immune cells influence bone function by releasing regulatory mediators that affect both bone formation (osteogenesis) and bone resorption (osteoclastogenesis), while immune system dysfunction can lead to pathological conditions including rheumatoid arthritis, bone destruction, osteoarthritis, and osteoporosis. Bone remodeling involves three primary cell types: osteocytes, osteoclasts, and osteoblasts. Osteocytes develop from osteoblasts and constitute the majority of bone cells, representing 90-95% of all bone cells. Osteoclasts are multinuclear cells responsible for bone degradation during normal remodeling and disease states, while osteoblasts (originating from pluripotent MSC) function as bone-forming cells that mineralize bone tissue and produce matrix proteins [24].

In this in vitro research, FDBB, dc-FDBB and DBBM are evaluated whether they cause physiological or non-physiological inflammatory responses by evaluating differences in IL-1 and IL-10 secretion levels between observation time groups and control groups. Research results in each control group and FDBB, dc-FDBB, and DBBM groups found no difference in IL-1 and IL-10 secretion levels on days 1, 3 and 7 which shows non-physiological inflammatory response patterns. This is consistent with research results by Pascual et al [25] which states that important concepts regarding inflammation or diseases mediated by IL-1 and IL-10, their severity is regulated at ligation production and activity levels, not at receptor levels. For example, IL-1 type receptors are expressed in all healthy individual cells and only rise 2-3 times when inflammation or disease occurs.

Besides, the above phenomenon can be caused by the following technical factors: non-physiological inflammatory response could be due to irritation or muscle inflammation factors during peripheral blood (hPBMCs) collection from volunteers. This is consistent with research results showing that IL-1 is required for inflammatory responses due to muscle tissue irritation induction but is not dependent on caspase-1. In sterile inflammation models, cell death and increased IL-1 precursors from macrophages or monocyte infiltration are present in research subject blood. Neutrophil infiltration is always followed by neutrophil proteases consisting of elastase, chymases, granzyme A, cathepsin G, and proteinase-3 [26]. Research results conducted reveal no difference in IL-1 and IL-10 secretion levels between control group and FDBB, dc-FDBB and DBBM groups in each observation (days 1, 3, and 7). This proves that FDBB, dc-FDBB and DBBM do not cause non-physiological inflammatory responses. Thus, it can be concluded that FDBB, dc-FDBB and DBBM are biocompatible.

## 5. CONCLUSION

The dc-FDBB scaffold showed a significantly increased secretion level of the pro-inflammatory cytokine Interleukin-1, indicating a tendency toward heightened immunogenicity. In contrast, the FDBB and DBBM scaffolds did not show significant increases in the secretion levels of either Interleukin-1 or Interleukin-10. Based on the statistical analysis of IL-1 and IL-10 secretion levels, no significant differences were observed among the FDBB, dc-FDBB, and DBBM scaffold groups. Ultimately, the FDBB, dc-FDBB, and DBBM scaffolds were found to be non-antigenic.

Future studies could include the examination of T cells, natural killer (NK) cells, and B cells in human peripheral blood mononuclear cell (hPBMC) cultures to determine their influence on the immunogenic response to the scaffolds. Expanding the range of immunogenic response indicators and including hPBMC cultures from individuals with different blood types

may also provide broader insights. Additional analysis using scanning electron microscopy (SEM) is recommended to examine the matrix structure of PBMCs and scaffold composition in greater detail.

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