CYTOTOXICITY TEST OF BOVINE DEMINERALIZED BONE MATRIX ON HUMAN MESENCHYMAL STEM CELLS USING THE MTT ASSAY METHOD

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ABSTRACT

The use of bone grafts in Indonesia continues to increase each year. Although Autograft is considered the gold standard in bone grafting, its use is often confronted with various challenges, similar to allograft. To address this issue, Bovine Demineralized Bone Matrix (DBM) can be considered as a substitute for bone grafts with the advantages of unlimited availability and more affordable costs. Currently, the Tissue Bank of Dr. Soetomo Hospital is developing bovine DBM, although there is no research yet on its potential toxicity.This study aims to evaluate whether bovine DBM has cytotoxic effects on human mesenchymal stem cells. In this experimental study, a total of 48 samples were involved, including a control group and two treatment groups (50% and 25%), each consisting of 16 samples. Mesenchymal stem cells were cultured and then treated with the addition of 50% and 25% DBM. Subsequently, cell viability was measured using the MTT Assay method.The collected data were processed by conducting normality and homogeneity tests and then analyzed using comparative tests with an independent t-test. The criteria for declaring cell toxicity were set at a viability of not less than 60% compared to the control group.The results of the MTT assay measurements showed that the mean Optical Density (OD) in the control group was 0.656 ± 0.021 (range 0.620-0.696), while in the treatment groups, it was 0.565 ± 0.022 (range 0.529-0.614) and 0.520 ± 0.022 (range 0.461-0.552), respectively. Statistically, the differences in OD between the control group and both treatment groups (50% and 25%) were significant ($p<0.05$). The average cell viability in both treatment groups was found to be more than 60%, indicating that Bovine Demineralized Bone Matrix is not toxic to human mesenchymal stem cells.

Keywords: Bone graft; mesenchymal stem cells; bovine demineralized bone matrix; viability; cytotoxicity

INTRODUCTION

The use of bone graft in Indonesia continues to increase each year, correlated with the high number of accidents and the rising prevalence of diseases that can lead to bone defects. Globally, it is estimated that around 2.2 million grafting procedures are performed annually (Werier, 2011). In the United States, the number of bone grafting procedures reaches 500,000 per year, and it is expected to double by 2020, a trend also observed in developing

countries like Indonesia (Winoto, 2010). According to the census data from the Diagnostic Center building at Universitas Airlangga in 2015, RS Dr. Soetomo reported 385 bone grafting procedures. The tissue-based approach is considered the best method for treating bone defects, involving the use of human tissue throughout the entire bone regeneration process.

Bone grafting is essential to support healing in various conditions, including delayed unions, nonunions, osteoectomies, arthrodesis, multifragment fractures, and to replace lost bone due to neoplasia or cysts. Although autogenous bone graft is considered the gold standard in bone grafting due to its histocompatible and non-immunogenic properties, its use is often hindered by longer surgery times, morbidity associated with bone harvesting, and limited availability (Bigham, 2008).

The use of allograft also presents challenges, such as the risk of residual infection, high costs, and donor availability. While allograft has the advantage of availability in various forms and sizes without sacrificing recipient tissue, its osteogenic potential is lower than that of autograft. Allograft is also associated with the risk of bacterial and viral contamination, as well as the ability to induce an immunological reaction that can hinder the bone healing process and lead to graft rejection.

Another commonly used alternative is xenogenic or heterologous bone graft, taken from different species and implanted into humans. Xenografts, such as coral, porcine, and bovine, can provide an unlimited supply if processed safely for human use. However, caution is needed in the use of bovine xenograft due to the potential transmission of zoonotic diseases such as bovine spongiform encephalitis. Although xenografts lose some osteogenic and osteoinductive properties, they provide good results and are cost-effective alternatives (De Long, 2007).

Demineralized Bone Matrix (DBM) is an osteoconductive and osteoinductive biomaterial extracted from cortical bone through an acid process (Gruskin, 2012). DBM contains a significant amount of protein from bone, with minimal calcium, inorganic phosphate, and debris content. DBM preparations in various forms, such as powder, solid composites, strips or sheets, and semi-solid paste, are widely used as degradable bone-filling devices (Holt, 2012). The use of Allogeneic DBM is limited due to difficulties in harvesting and selecting bone from human donors. Conversely, xenogeneic DBM can provide an unlimited supply and is safe for human use. Powdered DBM is more efficient for filling small bone defects (Block, 1995).

The use of xenogeneic DBM in Indonesia is expected to replace the use of autografts, which are generally expensive and limited in supply. Although RS Dr. Soetomo currently uses commercially available DBM products at a relatively high cost, the development of the hospital's tissue bank is underway to produce DBM.

METHODS

This research is an experiment conducted at the Institute of Tropical Disease (ITD), Universitas Airlangga. In this study, mesenchymal stem cell samples will be divided into two categories, namely the case group and the control group. In the case group, RSDS bovine DBM with concentrations of 50% and 25% will be introduced, while in the control group, it will not. Both groups will then be tested for viability using the MTT Assay, and the results will be analyzed.

In this study, data were analyzed using SPSS software version 19.0. Prior to the analysis, the collected data underwent a data cleaning process, including coding, tabulation, and then input into the computer. The data analysis process involved descriptive analysis and hypothesis testing. Numeric-scale data were described using

mean values and standard deviations. Meanwhile, categorical variables were presented in the form of frequency distribution and percentages. Hypothesis testing was conducted using the Two-Sample t-test.

RESULTS

The study on the toxic effects of xenogenic demineralized bone matrix on the viability of mesenchymal stem cells was conducted through the measurement of optical density using the MTT assay, with the following results

Table 1. The Research Results of Optical Density Data

Number	OD of Cell Control	OD Medium	OD Test 50%	OD Test 25%	Corrected OD Test 50%	Corrected OD Test 25%	Corrected OD of Cell Control
1	0.717	0.056	0.616	0.600	0.560	0.544	0.661
2	0.756	0.060	0.612	0.612	0.552	0.552	0.696
3	0.723	0.052	0.581	0.576	0.529	0.524	0.671
4	0.735	0.052	0.600	0.587	0.548	0.535	0.683
5	0.711	0.056	0.621	0.595	0.565	0.539	0.655
6	0.743	0.054	0.598	0.565	0.544	0.511	0.689
7	0.716	0.060	0.623	0.577	0.563	0.517	0.656
8	0.705	0.055	0.653	0.585	0.598	0.530	0.650
9	0.721	0.058	0.635	0.564	0.577	0.506	0.663
10	0.701	0.063	0.641	0.579	0.578	0.516	0.638
11	0.698	0.065	0.611	0.565	0.546	0.500	0.633
12	0.687	0.067	0.609	0.601	0.542	0.534	0.620
13	0.715	0.059	0.597	0.586	0.538	0.527	0.656
14	0.737	0.068	0.585	0.559	0.517	0.491	0.669
15	0.703	0.065	0.601	0.601	0.536	0.536	0.638
16	0.687	0.057	0.590	0.518	0.533	0.461	0.630
The corrected Optical Density (OD) is the measurement result OD minus the OD of the medium							

Table 2. Descriptive Data of the Research Samples

OD Post Correction	N	Min	Max	Mean ± SD
OD of Cell Control	16	0.620	0.696	0.656 ± 0.021
OD Test 50%	16	0.517	0.598	0.551 ± 0.021
OD Test 25%	16	0.461	0.552	0.552 ± 0.022

The MTT assay measurements in the control group (stem cells) yielded an average optical density measurement of 0.656 ± 0.021 (range 0.620 - 0.696), and in the test group (stem cells + xenogenic DBM), they were 0.551 ± 0.021 (range 0.517 - 0.598) and 0.552 ± 0.022 (range 0.461 - 0.552) for concentrations of 50% and 25%, respectively. The collected optical density data were then subjected to mathematical calculations to obtain the percentage values of stem cell viability post-treatment, using the following formula.

$$\%\ live\ cells\ (viabilitas) = \frac{treatment\ OD - OD\ medium\ (corrected\ treatment\ OD)}{OD\ of\ cell\ control - OD\ medium\ (OD\ of\ selected\ control\ cells)}$$

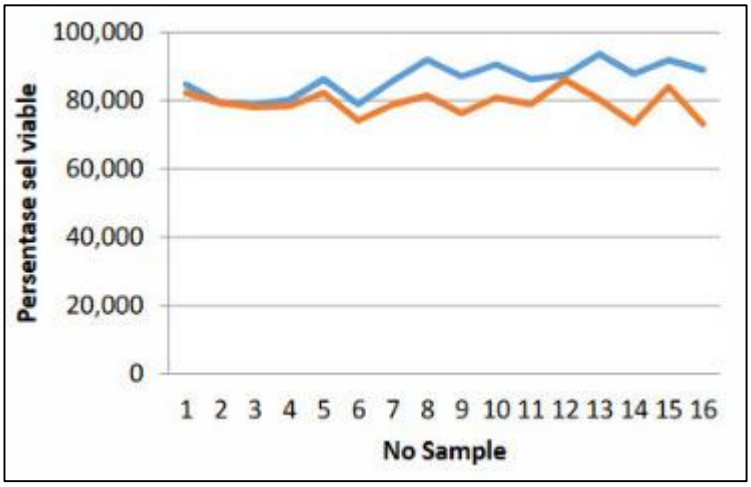

Figure 1. Percentage of viable cells in the treatment group graph. Number of cells in the 50% treatment group (blue line), meanwhile number of cells in t5he 25% treatment (orange line).

Table 3. Post-Treatment Stem Cell Viability Data

	Number of cells in the 50% treatment group	**% Viability**
OD of Control Cells	0.656	-
OD Test 50%	0.551	83.993
OD Test 25%	0.520	79.206

After xenogenic DBM administration relative to the control stem cells. It appears that both after the administration of 50% DBM and 25%, the viability of mesenchymal stem cells is above 60% (83.993% and 79.206%, respectively).

In this study, a comparative analysis was conducted by comparing the optical density values of the control cell group with the treatment group and comparing the optical density values between treatment groups (the OD values taken were post-correction).

Table 4. Comparison of Optical Density in Control Cells and Optical Density in Test Cells with 50% Concentration

Group	**N**	**Mean ± SD**	**Difference**	***p***
Cell Kontrol	16	0.656 ± 0.021	0.105	0.000
Test 50%	16	0.551 ± 0.021		

The measured optical density post-MTT assay in the control cell group after correcting for medium optical density was 0.656 ± 0.021 compared to 0.551 ± 0.021 in the test group with a 50% DBM concentration. This difference is statistically significant ($p < 0.05$), although clinically, there is not a significant meaningful difference between them (OD difference of 0.105).

Table 5. Comparison of Optical Density in Control Cells and Optical Density in Test Cells with 25% Concentration

Group	N	Mean ± SD	Difference	*p*
Cell Control	16	0.656 ± 0.021	0.136	0.000
Test 25%	16	0.520 ± 0.022		

The measured optical density post-MTT assay in the control cell group after correcting for medium optical density was 0.656 ± 0.021 compared to 0.520 ± 0.022 in the test group with a 25% DBM concentration, and this difference is statistically significant ($p < 0.05$).

Table 6. Comparison of Test OD at 50% and Test Cell OD at 25%

Group	N	Mean ± SD	Difference	*p*
Test 50%	16	0.551 ± 0,021	0.031	0.000
Test 25%	16	0.520 ± 0,022		

The measured optical density post MTT assay between treatment groups after correcting for medium optical density is 0.551 ± 0.021 for the test group with 50% DBM concentration, compared to 0.520 ± 0.022 for the test group with 25% DBM concentration. Statistically, this difference is significant ($p<0.05$), although clinically there is not a substantial meaningful difference between the two (OD difference of 0.031).

DISCUSSION

In this study, the viability of the treatment group was recorded to be lower compared to the control group. The decrease in viability in the treatment group may be attributed to the death of some mesenchymal stem cells after the treatment. The death of mesenchymal stem cells may be triggered by the presence of calcium still present in DBM, considered as a foreign substance capable of inducing oxidative stress on mesenchymal stem cells (Rodrigues et al., 2010). Stress on the mitochondria of mesenchymal stem cells can increase the production of Reactive Oxygen Species (ROS) by the mitochondria. Although an increase in ROS is a natural mechanism to eliminate pathogens and foreign substances, excessive ROS levels, due to their high chemical reactivity, can lead to cell death through irreversible peroxidation of lipids, amino acids, nuclei, and carbohydrates. Residual Calcium in DBM acts as a foreign substance that induces an increase in ROS, which then activates apoptosis signaling, leading to cell death. Activation of apoptosis signal-regulated kinase 1 (ASK1) triggered by ROS occurs through the activation of p38MAPK, a pathway responsible for cell death in the stem cell population (Shi et al., 2012).

The lower cell viability in the treatment group may also be due to the inability to increase the proliferation of mesenchymal stem cells. This could be related to the absence of BMP-2 content in DBM, preventing the activation of the MAPK cascade that plays a role in the proliferation of mesenchymal stem cells. As known, BMP-2 requires binding to BMPRs surface receptors to initiate the cell proliferation cascade. The binding of BMP-2 to residual minerals in DBM may also explain this phenomenon, as BMP-2 that is not released cannot bind to BMPRs (Rodrigues et al., 2010).

CONCLUSION

The demineralized bone matrix derived from cows (Bovine Demineralized Bone Matrix/DBM) exhibits toxic properties against human mesenchymal stem cells. This implies that the use of DBM in this context can lead to a decrease in the viability of mesenchymal stem cells and induce toxic mechanisms, such as an increase in Reactive Oxygen Species (ROS) and the activation of the apoptosis pathway, ultimately resulting in cell death. This conclusion highlights the potential negative impact of using DBM in interaction with human mesenchymal stem cells.

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