Analysis of the Mean Gray Scale and the Mandibular Thickness (Micro-CT) To Mandibular bone Density in Synthetic SCAFFOLD-hADMSC

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Abstract

Introduction. The concept of biofactor transplantation in porous and degradable materials as scaffolds is a place for regenerating new cell and bone tissue growth in the application of tissue engineering. Human Adipose Derived Mesenchymal Stem Cells (hADMSC) is a multipotent cell which has osteogenic, chondrogenic, and adipogenic potential. It also has somany similar characteristics with Bone Marrow stem cells (BMSCs) but hADMSC has higher proliferation rate compared to BMSCs.Inspite of this, another ability of biofactors is gene engineering. **Aim.** This study aimed to analyze mandibular bone density from the synthesis of scaffold-hADMSC with the mean gray scale and the mandibular bone thickness (Micro-CT).**Material and Methods.** This research involved several processes, namely synthetic scaffold manufacturing process, differentiation and characterization of hADMSC, and Micro-CT. **Results.** The results of the marked expression of the characterization of hADMSCand analysis of the mean gray scale and mandibular thickness (Micro-CT) to mandibular bone density in synthetic scaffold-hADMSC showed to be significant. **Conclusions.** Synthetic scaffold-hADMSC can increase mandibular bone density after Micro-CT analysis.

Keywords: *Micro-CT,Mesenchymal Stem Cell, syntheticscaffold*

Introduction

The concept of transplantation is biofactors (cells, genes and or proteins) which is an porous and degradable materials as scaffold.[1] Scaffold is a place for regenerating new cell and bone tissue growth in tissue engineering applications.[2] Human Adipose Derived Mesenchymal Stem Cells (hADMSC) is a multipotent cell which can differentiate into osteogenic, chondrogenic, and adipogenic. hADMSC has many similar characteristics of Bone Marrow stem cells (BMSCs) but it has a higher proliferation rate compared to BMSCs.[3] The development of bioceramic scaffold has so many benefits onits high porosity interconnection which the place for the growing of the new tissue enters and binds.[4] The process of bone regeneration and cartilage utilization of hADMSCare still less effective and its process must be combined with the using of scaffold and growth factors.[5] Bioceramic as a scaffold synthesis seeded with hADMSC is a concept of bone regeneration process in tissue engineering.

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Material and Methods

Animal Selection and Criterion Procedure

This research obtained using rat (Rattus norvegicus) weighing 200-300 gram, male rats, and aging 2-3 months years old. These rats were divided into five groups, such

as: the first and third groups were given scaffold Y-TZP, the second and fourth groups were given both scaffold Y-TZP and hADMSC, and the fifth group was a control group. There were two kinds of measurements in this research, the first was the looking time and the second was the variables that would be looked for.

Adipose Sampling Procedure

Adipose sampling was performed with an oval slice on the upper surface, remaining adipose on the inner surface together with muscles and other tissues, with an area of approximately $5cm^3$.

Isolation, Culture, Characterization, Differentiation Procedure, and Flowcytometry hADMSC

Isolation and Culture Expansion Procedure of hADMSC according to (Banyard et al., 2015) without modification until passage 4.[6] Differentiation of hADMSC to determine adipogenic and osteogenic according to (Jeon et al. 2016) without modification.[8] Flowcytometry is carried out according to the procedure from (Van Pham et al, 2016) without modification.[7,8,9]

The procedure for implanting synthetic scaffold in the mandibular bone of rat

The anesthesia procedure is carried out by administering ketamine HCL injection intramuscularly at a dose of 20 mg per kg body weight and xylazin premedication 3 mg per kg body weight intramuscularly and then wait until the anesthetic effect works.

Disinfection of the mandibular extra oral with 10% povidone iodine in the work area and covered with sterile cloth. Mandibular incisions on the left side and flap to muscle and periosteum are released from the mandibular bone surface. After that, drilling is done laterally in the ramus mandibular area with a diamond wheel contra angle low speed handpiece. Furthermore, implant material is synthetic scaffold, both with seeding hADMSC and without seeding hADMSC on the mandibular bone, then suturing using gut 5/0 atraumatic silk cut thread. For experimental animals in the control group the treatment of the mandibular bone only defects the bone by drilling. The experimental animals in normal controls were not treated and terminated at 8th week in experimental animals. After treatment, each experimental animal is given antibiotics and analgesics to avoid secondary infections, pain, and death. Post-implantation wounds are controlled every day. Similarly, daily control is carried out on the condition of the cage and food.

Procedure for taking research samples

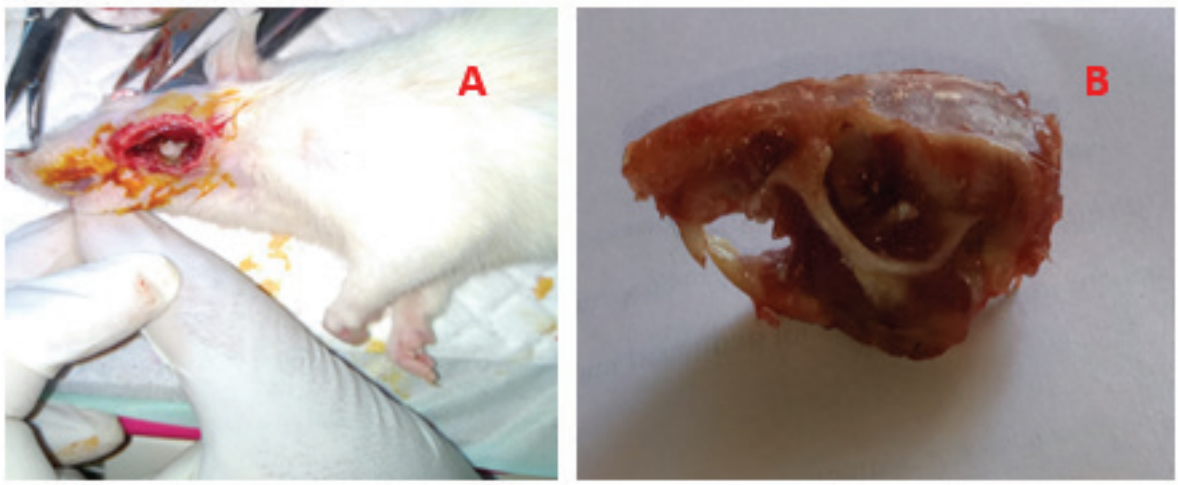

Figure 1.Procedure for sampling and treatment. A) Sacrificed methode and implantation of synthetic scaffold. B)Treatment sample.

After synthetic scaffold implantation at the end of the first week and 8th week of each treatment group and treatment control and normal control were taken 4 rats were sacrificed for the procedure of taking the research specimens. After decaputating process, the mandibular rat were cut in the control group and treatment with synthetics caffold implantation by cutting laterally included synthetic and soaked in a fixation solution of 10% buffered formaldehyde for a minimum of 3 days. The sample was then examined with Micro CT and incinerated at the the Faculty of Veterinary Medicine, Airlangga University.

Micro-CT examination procedure

The scanning procedure is carried out using a Micro-Computed Tomography (Micro-CT) device Bruker SkyScan 1173 High Energy Micro-CT.

Reconstruction image is a collection of 2D images in the lateral slice plane (x-y plane). Reconstruction is done by using the isotropic spatial resolution mode where the image voxel has a resolution of 28.15 microns, which also means that the thickness of the 2D image resulting from the reconstruction is 28.15 microns. This image stack contains complete information on the structure of 3D space, which can be visualized in the form of orthoslice or 3D volumetric visuals. Giving color codes can be done to reinforce differences in structure that are very useful for qualitative analysis. The analysis was then carried out on the sample by calculating the Mean of Grayscale Index, and the trabecular thickness according to the operational definition described above.[10]

Ethics

This study has been evaluated and approved by Committee Ethics Airlangga University Hospital with Number: 107/KEH/2018.

Results

Isolation, Culture Expansion, Characterization, Differentiation, and Flowcytometry of hADMSC phenotype

Based on the characterization analysis of hADMSC, MSCs on the expressions of CD 90 (97.61%), CD 73 (99.56%) and CD 105 (97.41%) were above 95%, while they on CD 14 (0.74%), CD 19 (0.83%), CD 34 (1.45%), CD 45 (1.42%) and also HLA-DR (0.84%) were below 2%.

The Micro-CT analysis

Figure 2.The Micro-CT analysis. A)2D image of Micro-CT orthoslice. B) 2,5D image of Micro-CT orthoslice. C) 3D image of Micro-CT orthoslice. D) analysis of MGI statistics between treatment groups in termination of the same week. E) MGI statistical analysis between groups 1 and 8 weeks. F) analysis of mandibular thicknessstatistics between treatment groups in termination of the same week. G) mandibular thickness statistical analysis between groups 1 and 8 weeks.

Table 1. Statistical Analysis of The Mean Grayscale Index (MGI)

Group	n	MGI			P value
		Mean±SD	Median	Min-Max	
Normal (KN)	4	88.5 ± 5.31	90.67b	80.60 - 92.07	
Defect One (K1)	4	56.69 ± 2.34	56.21a	54.55 - 59.77	
Defect + Scaffold 1 (P1)		85.63 ± 1.45	86.16b	83.50 - 86.70	
Defect + Scaffold + Stem Cell 1 (P2)	4	87.09 ± 2.31	87.55b	84.20 - 89.07	0.011*
Defect Eight (K2)	4	62.76 ± 4.88	65.25a	57.14 - 65.89	
Defect + Scaffold 8 (P3)	4	88.02 ± 0.88	88.37b	87.03 - 88.68	
Defect + Scaffold + SC 8 (P4)	4	88.39 ± 3.53	87.48b	85.60 - 93.00	

*Kruskal-Wallis test showed that P2 has a significant value with p=0.011 (α=0.05)

[a,b] denotes from Wilcoxon-Mann Whitney Test which [a,b] showed that there was no significant differences (α=0.05)

Figure 3.The graphic of descriptive statistic MGI

Table 1 and Figure 3 showed the data of Mean Grayscale Index (MGI) of each group was checked by normality test and the distribution of the data was not normal, so it must be analyzed using non-parametric test. From these non-parametric test, between the P1, P2, P3, and P4 there was no significant difference with KN, but the group K1 and K2 there was significant difference with KN. Between each group, K1 K2 K3 and K4 there was significant difference with K1 and K2.

Table 2. Statistical Analysis of Mandibular Thickness

Group	n	Trabecula Thickness			P value
		Mean±SD		Min-Max	
Normal (KN)	4	0.38 ± 0.07^{c}		0.31-0.45	
Defect One (K1)	4	0.08 ± 0.01^{a}		0.07-0.09	
Defect + Scaffold 1 (P1)		0.16 ± 0.06^{ab}		0.12-0.23	
Defect + Scaffold + Stem Cell 1 (P2)	4	0.18 ± 0.04^{ab}		0.15-0.24	0.001*
Defect Eight (K2)	4	0.20 ± 0.11^{abc}		0.13-0.32	
Defect + Scaffold 8 (P3)	4	0.21 ± 0.05^{abc}		0.17-0.27	
Defect + Scaffold + SC 8 (P4)	4	0.18 ± 0.03^{b}		0.14-0.20	

*Statistic Welch showed that P2 has a significant value with p=0.001 (α=0.05)

[a,b,c] denotes from multiple comparisons Games-Howell which [a,b,c] showed that there was no significant differences (α=0.05)

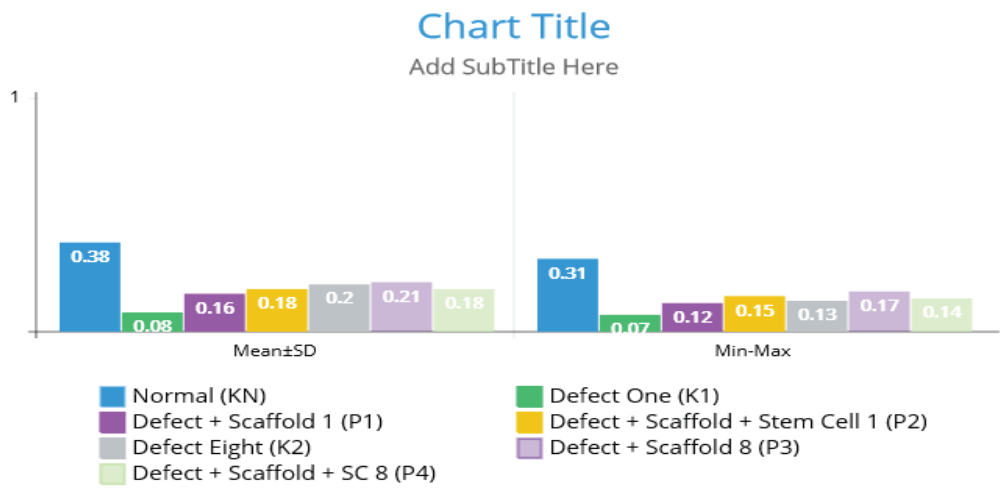

Figure 4.The graphic of descriptive statistic mandibular thickness

Table 2 and Figure 4 showed the statistical analysis of mandibular thickness using both Statistic Welch and multiple comparisons Games-Howell. The result of the mandibular thickness was normally distributed, so that it can be analyzed using parametric test using both Statistic Welch and multiple comparisons Games-Howell. After being tested using both parameters, it can be asked that between group K2 and P3 there was no significant difference, but for the group P1, P2, P3, and K2 there was no difference with groups P4, K1, and KN. But, there was significant difference between with groups KN, K1, and P4.

Discussion

The main problems that affect the process of success in an implant process consists of: 1. Inflammation, 2. Osteogenic, and 3. Osseointegration. In the first stage is the process of inflammation or inflammation considering the implant process is a process that is invasive so it may cause tissue damage that will spur the body's immune system to conduct repair or regeneration. One form of immunity is the release of cytokines due to the formation of Danger Associated Molecular Patterns (DAMPs). DAMPs can consist of 2 types, namely: High Mobility Group Boxes-1 (HMGB-1) protein and Adenosine Tri Phosphate (ATP). DAMPs are released and formed by degenerating body cells, causing sterile inflammation in the vertebrates and triggering the activation of the immune system and transcription factors. Activated immune cells are macrophages and neutrophils which further stimulate the formation of inflammatory factors (IL-1β and TNF-α). In addition to the inflammatory factors, the presence of proteases and free radicals also contribute to the formation of a secondary inflammation system in tissues facilitated by NF-KB which was initially in an inactive state to become active. Activation of NF-KB is caused by the synthesis of IKB, and activation of NF-KB in the next stage will trigger a bond with Toll Like Receptor-4 (TLR-4) so that there is a regulation of multiple-level network transcription factors, such as: IL-1β, TNF-α, IL-6, the enzyme Cyclooxygenase 2/COX-2, Matrix Metalloproteinase, and transcription factors and other signals related to osteoblast synthesis (Runx2 / Run-Related Factor Transcription-2, Osterix, Alkali Phosphatase / ALP enzymes, Ephrine specifically Ephrine 4, Caderin specifically CDH2 and CDH10b, Osteoprotegerin / OPG, Wnt LPR5 / 6). Inflammatory factors that are formed in the initial phase are also related to the stem cell material used in this study, namely: by using human Adipose Mesenchymal Stem Cells (hADMSCs) which have a common pathway in the activation process, namely through the CXCR 4 chemokine with the CXCR 4 also present in the inflammatory phase which is influenced by the chemokine CXCL12. The CXCR4 chemokine is also related to one of stem cell characterization, which is homing characterization on stem cells.

After dealing with inflammatory phase, there are several types of transcription factors which can originate from the transcription factors themselves and from the transcription factor signals which further enhance osteoblast synthesis. Increased osteoblast activity will cause an acceleration of bone formation, which is in accordance with the results of statistics conducted on animal experiments that have a significant value with a p value of 0.001 which turned out to be smaller than the α value of the statistical test 0.05. After a lot of osteoblast synthesis, both from the conduction of osteogenic transcription factors and from the HADMSCs stem cells, there is further collagen synthesis, specifically type I collagen which functions in the process of biomineralisation. This biomineralization process is an important process which is controlling and forming Hydroxy Apatite.

The final stage of the implantation process is the osseointegration process which means that the process of integration between the implant material and surrounding bone tissue. This is controlled by caderin which is a protein located at the adhesive junction between cells involved in the process of morphogenesis, tissue formation, and tissue integrity. Caderin works in two ways, namely: in the osteogenic process and endosteal niche which has the same end result, namely the increase in osteoblast cells caused by caderin, specifically CDH-2, CDH-10b will increase the occurrence of Wnt complex with β-catenin and related with differentiation of hAMDSCs into osteoblast cells.

The next step is to examine the thickness of the bone with a device called a Micro-CT which provides information about terminology for the measured parameters and related units, and further analysis has the potential to be done with this technology. Shortly, qualitative and quantitativeanalysis, including basic image processing (selection and VOI threshold) and measuring of several morphometric variables (total VOI volume, object volume, percentage of total volume, total VOI surface, object surface, object surface / volume ratio, object density surface , structure thickness, structure separation, total porosity).[10] The micro-CT observation was carried out by 2D image analysis; 2.5D; 3D. In 2D and 2.5D images there are noise / artifacts (radioopaqe spans) in the area around synthetic scaffold in all treatments. This is caused by the density of material from synthetic scaffold after sterilization using gamma rays. Synthetic scaffold shrinks from its original form before sterilization using gamma rays. Sterilization is needed to avoid contamination of synthetic scaffold especially for preparing seeding with hADMSC.At MGI it was found that mandibular bone density was significantly greater in the treatment of scaffold-hADMSC synthesis compared to the treatment of scaffold synthesis without hADMSC with reference to normal control. At the mandibular bone thickness it was also significantly clear that the mandibular bone density was greater in the treatment of scaffold – hADMSC synthesis compared to the treatment of scaffold synthesis without hADMSC, the normal control group as standartization of mandibular bone density.The hypothesis with sintesis scaffold-hADMSC in tissue engineering is that it has a marked osseointegration capabilities comparedsintesis scaffold withouth ADMSC. High power and osseointegration acceleration can reduce implantation failure. In the future, sintesis scaffold-hADMSC made a tissue engineering in dental implantation and orthopedics.

Conclusion

In conclusion,synthetic scaffold-hADMSC can increase mandibular bone density after Micro-CT analysis.

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