LAPORAN AKHIR TAHUN
INTERNATIONAL RESEARCH COLLABORATION AND SCIENTIFIC PUBLICATION (KLN)

OPTIMALIZATION THE POTENCY OF ANTI-OSTEOPOROSIS OF APIS DORSATA HONEY FOR THE NEW NATURAL COMPOUND PREPARATION TO PREVENT OSTEOPOROSIS

TAHUN KE – 1 DARI RENCANA 3 TAHUN

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KEMENTERIAN RISET, TEKNOLOGI, DAN PENDIDIKAN TINGGI
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Summary

Osteoporosis (OP), defined as low bone mass leading to increased fracture risk, is a major health problem. In Indonesia one from four female had OP risk (50-80ys). The costs of osteoporotic fracture–related morbidity and mortality will burden the Indonesian healthcare system. Treatment of high-risk patients with antiresorptive medications such as bisphosphonates. For postmenopausalosteoporosis, the main treatment is estrogen replacement therapy (ERT). Despite its effectiveness, ERT, however, can cause many adverse effects.

Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the micronutrients of greatest importance are calcium and vitamin D. Calcium has been shown to have beneficial effects on bone mass at all ages, although the results are not always consistent cause depend on the capacity of Calcium absorption. The absorption of Calcium will increase as the amount of honey taken was upped.

Indonesiais the top second biodiversity in the world which has 7 species of bees while Honey is one of their product. Honeyas alternative treatment that is rich in antioxidant and also an anti-inflammatory effect can be given to replace theconventional ERT. Apis Dorsata honey is one of the best options available as it contains antioxidant as well as exerting anti-inflammatoryeffect which can act as a free radical scavenger, reducing the oxidative stress level as well as inhibiting proinflammatory cytokine.

This will result in survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone loss. Hence, Apis Dorsatahoney can be used as an alternative treatment of postmenopausal osteoporosis with minimal side effects.

The aim of this study are to find the new natural compounds for up of absorption Calcium response, to determine the suppressive mechanism of OP by new natural compound and to confirm the effect of new natural Honey compounds in animal experiment.

In three years, the research project will be conducted by two institutions, Faculty of Dental Medicine Universitas Airlangga-Indonesia and Faculty of Biosciences & Medical Engineering Universiti Teknologi Malaysia (UTM)-Johor Bahru, Malaysia.

At the first year we will focus on microstructure of bone effect by Apis Dorsata Honey. Total volume, bone volume, bone volume density, trabecular thickness, trabecular spacing, trabecular number, bone surface, cortical thickness, bone mineral content and bone mineral density are determined by μCT and FESEM analysis are used to evaluate the microarchitecture and porosity of bone tissue influence by Apis Dorsata honey.

Second, the experiment will focus on biomolecular and host respon by survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone loss to evaluate the bone tissue activity.

On the third year this project will be finished by optimizing preparat of Apis Dorsata Honey preparation as Drug patent also stability assesment material.Partner of this project had high experience on bone research and frequenly on publishing with Q1, Q2, ISI Index (113 paper) Scopus Index (163 paper) also owner some of invention (32 patents granted; 28 patend pending); copyrights (36); Impact Factor (229,854); h Index (15); books (6).

That`s why by this collaboration research, we expect the new natural compounds will be discovered. Furthermore, we confident submit the result of this project for international publication every years. The target journal is Journal of Material Science. The expected result will be gained as soon as possible and the new preventive medicine for OP will be created for human being.

PREFACE

All the praises and gratitude of the researchers offered to the presence of the Almighty God, for all His grace, so that researchers can complete this research in the hope of contributing knowledge about the use of Honey-based natural ingredients-based medicaments that are the result of Indonesia's natural resources. This research is also aimed at reducing the dependence on imported medicaments in osteoporosis management. The hope is that the Indonesian people will get bone health services at a more affordable cost.

During the completion of this research, researchers have received a lot of guidance, financial support, direction and assistance, both in the form of knowledge and moral support from the government of the Republic of Indonesia through the granting of Kemenristek grants for Basic Research Schemes (Foreign Cooperation Research). On this occasion, the researchers would like to express their greatest gratitude to:

1. Mr. Joko Widodo as President of the Republic of Indonesia
2. Prof. H. Mohamad Nasir, Ph.D., Ak as Minister in the Ministry of Technology and Higher Education Research of the Republic of Indonesia which has given researchers the opportunity to complete this research
3. Prof. Dr. Mohammad Nasih, SE., Mt., Ak., CMA as the Chancellor of Airlangga University who has given researchers the opportunity to work.

Researchers realize that this research still has many shortcomings, but hopefully this research will benefit the Indonesian people.

Surabaya, November 2018

Researcher

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Chapter 1.
Introduction

Osteoporosis (OP), defined as low bone mass leading to increased fracture risk, is a major health problem. In Indonesia one from four female had OP risk (50-80ys). The costs of osteoporotic fracture–related morbidity and mortality will burden the Indonesian healthcare system. Treatment of high-risk patients with antiresorptive medications such as bisphosphonates. For postmenopausalosteoporosis, the main treatment is estrogen replacement therapy (ERT). Despite its effectiveness, ERT, however, can cause many adverse effects. Osteoporosis also to be a major public health threat for almost 44 million US men and women aged 50 and older, or 55% of the population in that age range (1). In fact, 1 in2 women and 1 in 4 men over the age of 50 will fracture at some point in their lifetime. The costs to the healthcare system associated with osteoporotic fracture are17 billion dollars annually (2), with each hip fracture having total medical costs of $40 000. Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the nutrients of greatest importance are calcium and vitamin D.

Indonesia are the top second biodiversity in the world which has 7 species of bees which produce 2000 ton honey per year (Novandra and Widnyana, 2013). Seventy percent from the whole amount of honey production in Indonesia are honey of Apis dorsatawhich known as jungle honey (Hadisoesilo, Kahono and Suwandi, 2011).

A previous study has shown that administration of honey from species of Apis dorsata could restore the morphology of the tibia bones of ovariectomized rats (Zaid et al., 2010). Honey decreased serum collagen type I concentration and femoral RANKL and NFATc1 mRNA expression (Katsumata et al., 2015). Flavonoid such as quercetin, rutin, and kaempferol are property of honey. The mechanism of active compound of honey, flavonoid, is remained unclear. It has 5 possible mechanisms to protect bone strength. The first mechanism is by reduce bone loss via antioxidant activity, mitigate as anti-inflammation and trough osteoimmunological action and it has potential of pro-osteoblastic and anti-osteoclastic activity (Đudarić et al., 2015). The possibility statement in the review are indicate that the mechanism remained unclear. However, the beneficial of honey in bone health also in implant for bone fracture without exception mandible as bone of jaw. The purposes of this study is to evaluate the effect of anti-osteoporosis activity of Apis dorsata

honey in ovariohysterectomized rats and to make sure the mechanism effect of honey Apis dorsata. The expectation of this study is the effectiveness of Apis dorsata honey as anti-osteoporosis and in zirconia implant seeded with human adipose derived stem cells for bone fracture. Furthermore, of this study is to give contribution in the form of international scientific publication either conference or journal.

The study will be design in three years. At the first year for UTM portion, we will focus on microstructure of bone effect by Apis Dorsata Honey. Total volume, bone volume, bone volume density, trabecular thickness, trabecular spacing, trabecular number, bone surface, cortical thickness, bone mineralcontent and bone mineral densityare determined by μCT and FESEManalysis are used to evaluate the microarchitecture and porosity of bone tissue influence by Apis Dorsata honey. For Indonesia portion focus in serum assessment.

Second, the experiment will focus on biomolecular and host respon by survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone lossto evaluate the bone tissue activity.

On the third year this project will be finished by optimizing preparat of Apis Dorsata Honey preparation as Drug patent also stability assesment material.

UTM-Unair had good comunication and collaboration. Before this project, UTM-Unair also had project collaboration in 2017 with focus in bone regeneration research. UTM as partner had experience especially on bone research with many paper and book published.

Collaboration in this project will advantage one each other. For Indonesia side equipment and tools like μCT and FESEM are limited. Only Institute Teknologi Bandung had and expensive for assesment. UTM also had software especially for analyzing microstructure bone and we don't have it. The other side, our partner of this project had high experience on bone research and frequenly on publishing with Q1, Q2, ISI Index (113 paper) Scopus Index (163 paper) also owner some of invention (32 patents granted; 28 patend pending); copyrights (36); Impact Factor (229,854); h Index (15); books (6).

We confident to publish the research output of every year. The publication will be presented in international conference and submit for international publication indexing ISI-Scopus. The target jounal is Jounal of Materials Science/Journal of Tissue Engineering and Regenerative Medicine, Journal of Dental Research/ Journal of European Cell and Material.

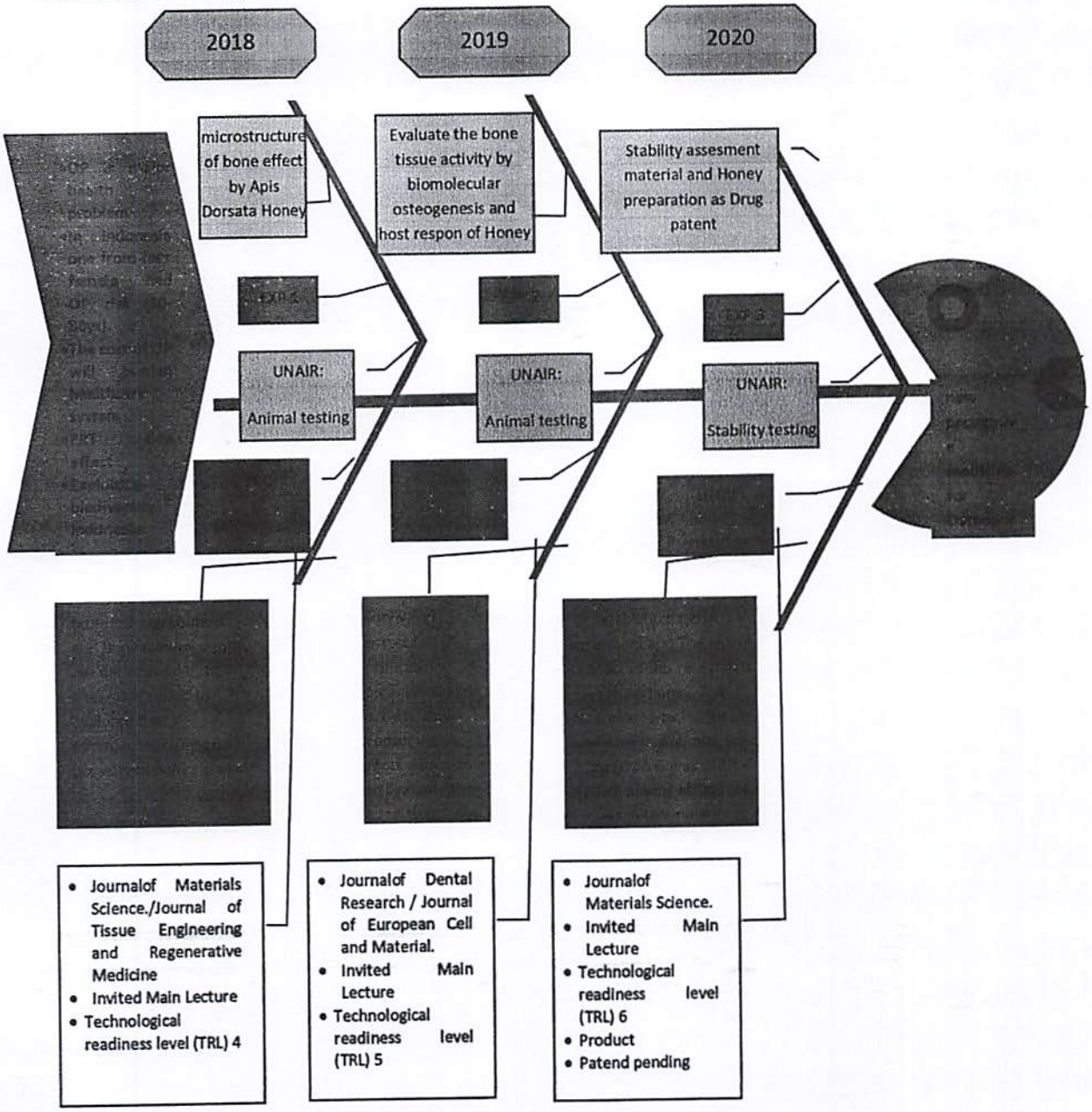
Another advantage of doing this collaborative research is continuing the previous study that has been started by the researcher from two institutions. The end of this research also to get new natural compound for preventing Osteoporosis.

Therefore, it is very easy to start the experiment immediately, the expected result will be gained as soon as possible and the new preventive medicine for Osteoporosis will be created for human being.

Chapter 2.

Literature Review

2.1 Fish bone diagram



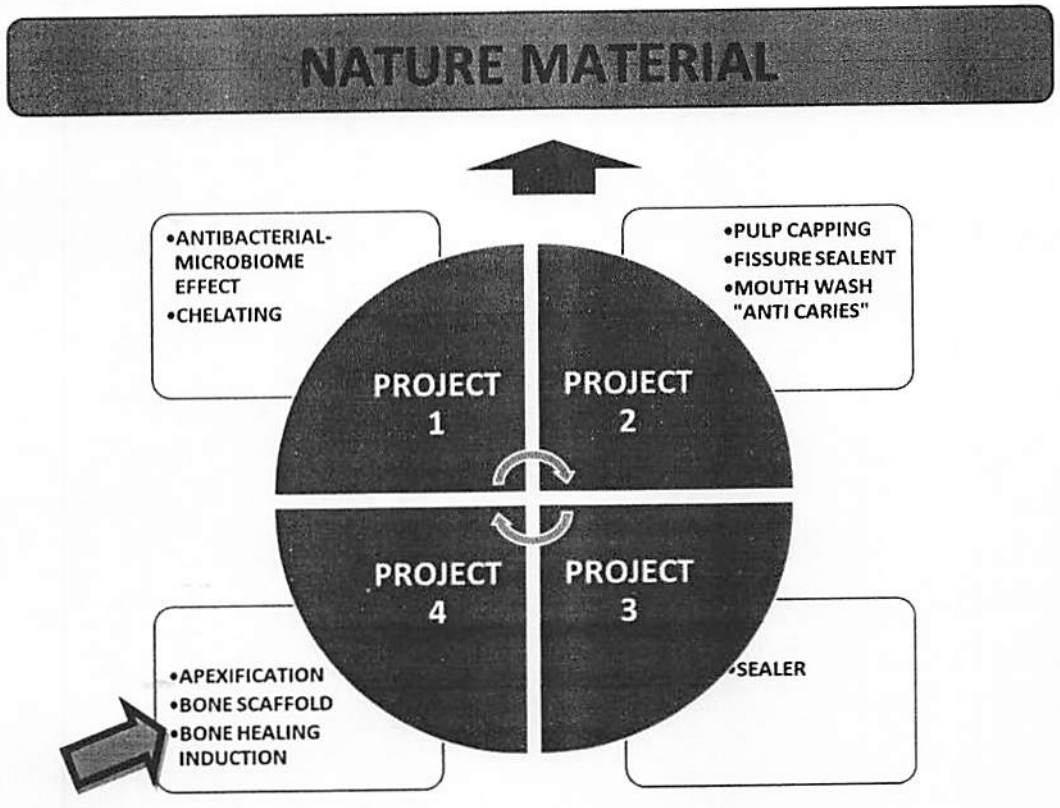
Osteoporosis (OP), defined as low bone mass leading to increased fracture risk, is a major health problem. In Indonesia one from four female had OP risk (50-80ys). The costs of osteoporotic fracture–related morbidity and mortality will burden the Indonesian healthcare system. Treatment of high-risk patients with antiresorptive medications such as bisphosphonates. For postmenopausalosteoporosis, the main treatment is estrogen replacement therapy (ERT). Despite its effectiveness, ERT, however, can cause many adverse effects. Osteoporosis is a accompanied by microarchitectural deterioration of bone tissue, which leads to an unacceptable increase in the risk of skeletal failure (fracture). Osteoporosis and low bone mass are currently estimated to be a major public health threat for almost 44 million US men and women aged 50 and older, or 55% of the population in that age range (1). In fact, 1 in2 women and 1 in 4 men over the age of 50 will fracture at some point in their lifetime. The costs to the healthcare system associated with osteoporotic fracture are17 billion dollars annually (2), with each hip fracture having total medical costs of $40 000. Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the nutrients of greatest importance are calcium and vitamin D. Numerous studies have shown that higher calcium intake at various ages are associated with higher bone mineral density compared with the bone mass of those with lower calcium intakes (3). In older postmenopausal women, the benefits of vitaminDand calcium supplementation in preventing bone loss, decreasing bone turnover, and decreasing nonvertebral fractures are clear (4). An inadequate intake of either calcium, vitamin D, or both will influence calcium-regulating hormones. A deficiency of either calcium or vitamin D will result in reduced calcium absorption and a lower concentration of circulating ionized calcium. When this occurs, parathyroid hormone (PTH) secretion is stimulated and there is a resulting increase in PTH levels. The cumulative D nutrition (secondary hyperparathyroidism), is an increase in bone remodeling leading to significant loss of bone and an increased fracture risk while treatment with implant. Vitamin D supplementation, often in combination with calcium, appears to reducethe degree of secondary hyperparathyroidism associated with poor nutrition.

Adequate nutrition plays a major role in the prevention and treatment of osteoporosis; the micronutrients of greatest importance are calcium and vitamin D. Calcium has been shown to have beneficial effects on bone mass at all ages, although the results are not always consistent cause depend on the capacity of Calcium absorption. The absorption of Calcium will increase as the amount of honey taken was upped.

Indonesia is the top second biodiversity in the world which has 7 species of bees while Honey is one of their product. Honeyas alternative treatment that is rich in antioxidant and also an anti-inflammatory effect can be given to replace theconventional ERT. Apis Dorsata honey is one of the best options available as it contains antioxidant as well as exerting anti-inflammatoryeffect which can act as a free radical scavenger, reducing the oxidative stress level as well as inhibiting proinflammatory cytokine.

This will result in survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone loss. Hence, Apis Dorsatahoney can be used as an alternative treatment of postmenopausal osteoporosis with minimal side effects and influencing with Zirconia implant seeded with human adipose derived mesenchima stem cells.

2.3 Road Map



2.4 Contribution

a. New natural compound for preventing Osteoporosis
b. Improvement of Indonesian natural sources
c. Decrease health unit cost for Osteoporosis
d. Increase life quality Indonesian people especially women
e. Impact of supplemen on implant Zirconia for bone fracture

Chapter 3.

The Aim and benefit

3.1 Tujuan

The aim of this study are to find the new natural compounds for up of absorption Calcium response, to determine the suppressive mechanism of OP by new natural compound and to confirm the effect of new natural Honey compounds in Zirconia implant seeded with human adipose derived mesenchimal stem cells on animal experiment.

In three years, the research project will be conducted by two institutions, Faculty of Dental Medicine Universitas Airlangga-Indonesia and Faculty of Biosciences & Medical Engineering Universiti Teknologi Malaysia (UTM)-Johor Bahru, Malaysia.

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Second, the experiment will focus on biomolecular and host respon by survival of osteoblasts, reduced osteoclastogenic activity, and consequently, reduce bone lossto evaluate the bone tissue activity.

On the third year this project will be finished by optimizing preparat of Apis Dorsata Honey preparation as Drug patent also stability assesment material

By this collaboration research, we expect the new natural compounds will be discovered. The expected result will be gained as soon as possible and the new preventive medicine for OP will be created for human being.

In three years, the research project will be conducted by two institutions, Faculty of Dental Medicine Universitas Airlangga, Indonesia and Faculty of Bioscience and Medical Engineering-UTM Johor, Malaysia. For time plan schedule, the first-year animal testing experiment will be done in Indonesia, meanwhile for assesment in Malaysia, the second and the third year will be finished in two countries, Indonesia and Malaysia.

Chapter 4

Materials & Methods

4.1 Research mapping

The research project will be conducted in three steps. The aim of step one is finding effect of the implant component in stem cells and combining with new natural medicine in postmenopausal osteoporosis in animal laboratory as a pre-clinical study. In the step two the experiment will focus on osteogenesis host respon by Apis Dorsata honey. At the last step the project will be finish by confirming Honey preparation as Drug patent and Stability assesment material combining with implant

Exp. 1 Biocompatibility of Yttria-Tetragonal Zirconia Polycrystal Seeded with Human Adipose Derived Mesenchymal Stem Cell

Y-TZPS Manufacturing Process

In this research, scaffolds used were made in a dental laboratory. Scaffold samples used were taken from Y-TZP [Vita YZ Interne Untersuchungen, VITA F & E: VITA Zahnfabrik H. Rauter GmbH & Co. KG Ressort Forschung und Entwicklung Spitalgasse 379713 Bad Säckingen Dipl.-Ing] [11]. Y-TZP have grooves and holes in their rod section with a Y-TZP size (Ø = 2.9mm, P = 3mm). In the manufacturing of Y-TZPS, sterilization was carried out using gamma cell radiation at Irradiation, Electromechanics, and PAIR-BATAN Instrumentation Center in Jakarta.

XRD Examination

Samples of Y-TZP were made in the forms of fine crystalline powder with a minimum size of 5 mg. To conduct XRD examination, [XRD D8 Focus, The Bruker Corporation (NASDAQ: BRKR), Billerica, Massachusetts, USA] and HCB were taken from male resection maxilla patients who were trafficking victims. Crystal powder that had been pressed into sample container, had a smooth surface, and was resistant to the samples at a 45-degree angle. A small amount of solid sample by depositing the substrate on a thin film placed on a sliding glass from the microscope, then the intensity of the beam is reduced from 40 KV and 40mA to 30kV and 30mA.

Log XRD analysis

The log must be filled in to operate the XRD before starting the test to be recorded. The XRD operation aims to check the alarm lights on the right side of the. Next, the XRD spreadsheet log on the desktop was filled in. KV setting, then was conducted by increasing the addition of 10kV at 30 seconds to 40kV. Subsequently, <set> button was pressed after every change. An mA setting of 5-10 then was carried out with an addition of 10mA to 40mA. Afterwards, a permanent parameter file was created for automatic scanning stored in the XRD wizard.

Adipose Sampling Procedure

Adipose or body fat tissue samples taken from participants with elective indications and no medical complications at Universitas Airlangga Hospital, Surabaya in accordance with certain criteria, such as pregnant women who underwent the birth process by cesarean section or Cesarean section due to abnormal fetus location

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 5 cm3. Adiposa then was put into a transport medium and taken to the Research and Development of Stem Cell Universitas Airlangga Laboratory (RDSCUAL), Surabaya for hADMSC isolation, cultural expansion, and TT.

Those Adiposa were washed with PBS solution, those adipose was cut and chopped until smooth and then were added by collagenase enzyme. Afterwards, they were soaked and incubated at 370 C for 45 minutes. It was added to the pellets, and planted on a 10 cm plate until the cells were attached to the base of each petri and labeled with the patient's identity in the form of the name and processing date, and then incubated in CO2 incubator.

Isolation and Culture Expansion Procedure of hADMSC

Isolation of hADMSC was using lipoaspirate enzymatic to separate SVF. Cells, on the other hand, are known consisting of stroma, progenitor SCs, WBCs, ECs, periocytes, and RBCs. Isolation and Culture Expansion Procedure of hADMSC according to (Banyard et al., 2015) without modification until passage 4 (12).

Characterization and Differentiation of hADMSC

Phenotype Characterization and differentiation of the MSC phenotype in hADMSC culture were conducted in two types of immunostaining methods, namely immunocytochemical staining and flowcytometry test (FT). Identification of hADMSCs was carried out at the SCIL of PT. Kalbe Farma Tbk with MSC phenotype kit after the fourth passage. Cells that have adipogenic, osteogenic, or chondrogenic properties are oil red-O staining (OROS) for adipogenic and Alizharin staining for osteogenic by dripping lipids and then fixed with 4%

formalin (13). Differentiation of hADMSC to determine adipogenic and osteogenic according to (Jeon et al. 2016) without modification (20).

Flowcytometry

Flowcytometry was performed to make monolayer cells change into single cells through trypsination process. After that, Flowcytometry is carried out according to the procedure from (Van Pham et al, 2016) without modification (14, 20 , 21).

hADMSC Seeding Procedure on Y-TZPS

In Y-TZPS, hADMSCs were hatched by immersing them in DMEM / F12 medium for 1 day. The old medium, then was removed and replaced by the new one. After one day, the Y-TZPSs were put into 24 culture wells (M24) with 2 x 106 cells (200 µL/well). Those wells, then were incubated for 1 hour at 370 C with 5% CO2. In the well the medium was added as much as 1.3 mL / well and incubated with 5% CO2 at 370 C. Periodically, the tubes were rocked to mix cells with the suspensions and Y-TZPSs. The cell-coated Y-TZPSs then were ready in the next 3 days for SEM.

Observation of Y-TZPS Seeded with hADMSCs Cells that had been cultured into the Y-TZPSs were fixed with 2% glutaraldehyde solution for 2 hours at 400 C, then washed with PBS solution 3 times for 5 minutes, replaced with 1% OAS for 1 hour at 400 C, and washed with PBS again. After that, they were dehydrated with alcohols from low to absolute levels for 15 minutes each, then replaced with absolute AAL as a preservative until dry time. Next, Y-TZPS were dried with CPD. They then were affixed to the stub by using special glue and coated with pure gold with a vacuum evaporator fixation tool. The Y-TZPSs then were ready to be observed and photographed with SEM at the DME, FIE, ITS, Surabaya.

TT on Y-TZPS and HCB

TT on Y-TZPS and HCB against hADMSC cell culture, trypination of a petri plate containing 2.5 x 106 cells was carried out. Next, resuspension was conducted in DMEM / F12 medium and centrifuged. Afterwards, the Pellets are planted into M96 of 5 x 104 cells / well, then incubated with 5% CO2 at 370 C for 24 hours. When 80% of the growth was obtained, the Y-TZPS were added to ½ parts of the wells. Subsequently, 100 µL of DMEM / F12 medium was added, and incubated with 5% CO2 at 370 C for 20 hours. After that, the wells were put in MTT as much as 5 mg / mL (25µL / well), then incubated for 4 hours, and observed under an inverted microscope. The Y-TZP and medium, then were discarded, and sDMSO was added into each well as many as 200 µL. Color changes in those wells were then read by the MTT Assay with Elisa Reader at a wavelength of 595 nm.

Ethics

This study has been evaluated and approved by RSUA (Airlangga University Hospital) Ethics Committee, Surabaya Indonesia with Ethical Clearance Number: 107/KEH/2018

Exp. 2 Effect of honey on fracture defect combining with implant seeded with human adipose derived mesenchimal stem cells

Chapter 5.

Result And Achievement Of Output

The manufacturing of Y-TZPSs and mineral analysis In the manufacturing of Y-TZPSs, there was ad distortion so that they did not only experience shrinkage in both dimension and density of their material, but their color also changed into browner than their original white color. These changes, as a result, had an impact on both the material density and the Y-TZPS density (Figure 1A). To know porosity in the scaffolds, magnification in using an SEM was required. The results of the mineral analysis with XRD examination were illustrated by a graph of Y-TZP, HCB, and a combination of both (Figure 1B). In Y-TZP, there was a high and sharp peak on the graph of material analysis with XRD examination. The Y-TZP graph was also known to have a low frequency. Similarly, in HCB there was a sharp peak with low frequency. Thus, to see their differences, XRD examination was performed on Y-TZP minerals combined with HCB minerals. The results showed that the height of the peak in Y-TZP was higher than that in HCB. Coefficient of thermal expansion had been determined by XRD measurement (15). Characterization and differentiation of hADMSC phenotype Isolation and Culture Expansion on hAMDSC of passage 1 was observed on the 3rd day followed by passages 2, 3, and 4 respectively until day 12. In passage 4, the cell density increased and was ready for both the adipogenic and osteogenic differentiations (AD and OD) of hADMSCs and the characterization of hADMSCs (Figure 2). Based on results of the ADA of hADMSC with OROS, there were more vacuoles than in cell controls. This shows that hADMSCs have properties that can be used for AD. Whereas the osteogenic differentiation analysis of hADMSCs with alizharin staining, it is indicated that hADMSCs have properties that can be differentiated towards OD by brownish vacuoles. Based on the CA 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%.

Observation Analysis (OA) on Y-TZPS with SEM

Results of OA on Y-TZPS using SEM with magnifications of 50x, 2500x, 5000x, and 7000x showed porosity in Y-TZPS. Meanwhile, results of the OA on Y-TZPS seeded with hADMSCs using SEM showed that there were cells attached to the surface of Y-TZPS seeded and fixed (Figure 4).

Toxicity Test

Results of the TT on Y-TZPSs and HCB in 96M revealed that both Y-TZPSs and HCB were not toxic to hADMSC.

Table 2 showed that Y-TZPSs seeded with hADMSCs had a higher percentage of LCs is 97% compared to HCB with hADMSCs (89%). In Figure 4, the results of the TT on HCB, Y-TZPSs, and CCs indicated that cells could live on HCB both before and after MTT Assay.

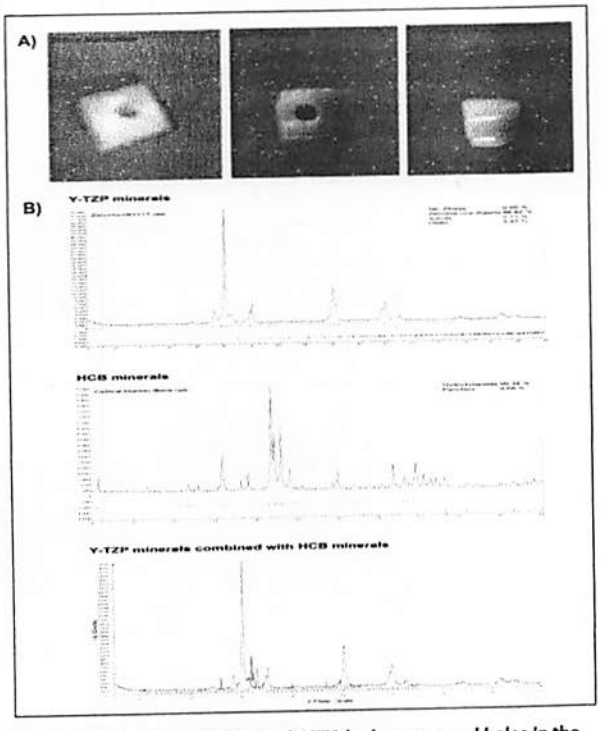

Figure 1. Distortion of Y-TZPSs. A)Y-TZP had grooves and holes in the rod section with size (Ø = 2.9mm, P = 3mm). B) mineral analysis with XRD examination. The peak on the graph of material analysis with XRD examination of Y-TZP minerals, HCB minerals, and Y-TZP minerals combined with HCB minerals

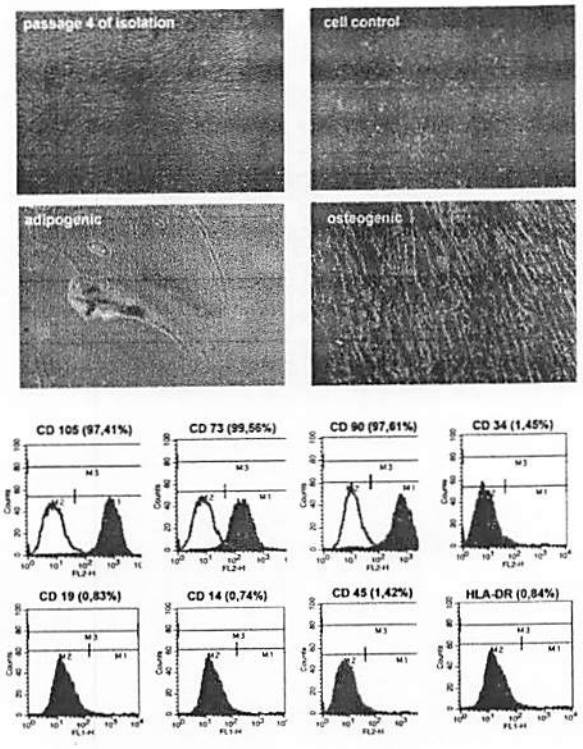

Figure 2. Isolation, differentiation, characterization and Graph Characterization Analysis (CA) of hADMSC. Adipogenic differentiation analysis (ADA) of hADMSCs with oil red-O staining (OROS). Osteogenic differentiation analysis (ODA) of hADMSCs with Alizahrin Staining. CA by immunocytochemical staining and flowcytometry test (FT).

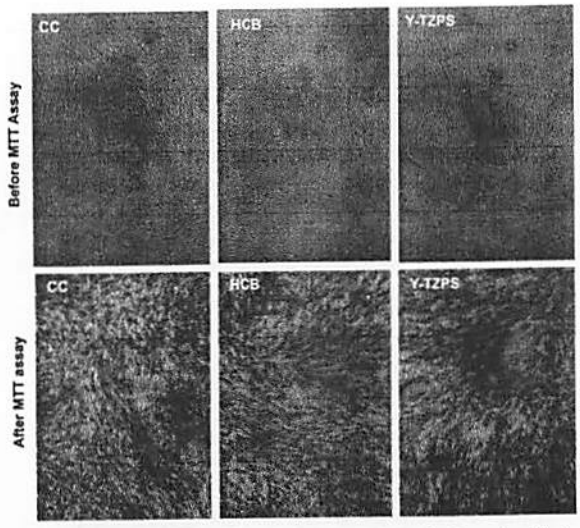

Figure 3. Observation Analysis on Y-TZPS by Scanning Electron Microscope (SEM). Y-TZPS 50x, 2500x, 5000x, and 7000x, (f) Y-TZPS seeded with hADMSCs 50x, 350x, 2500x, 5000x and 7000x. and Y-TZPSs

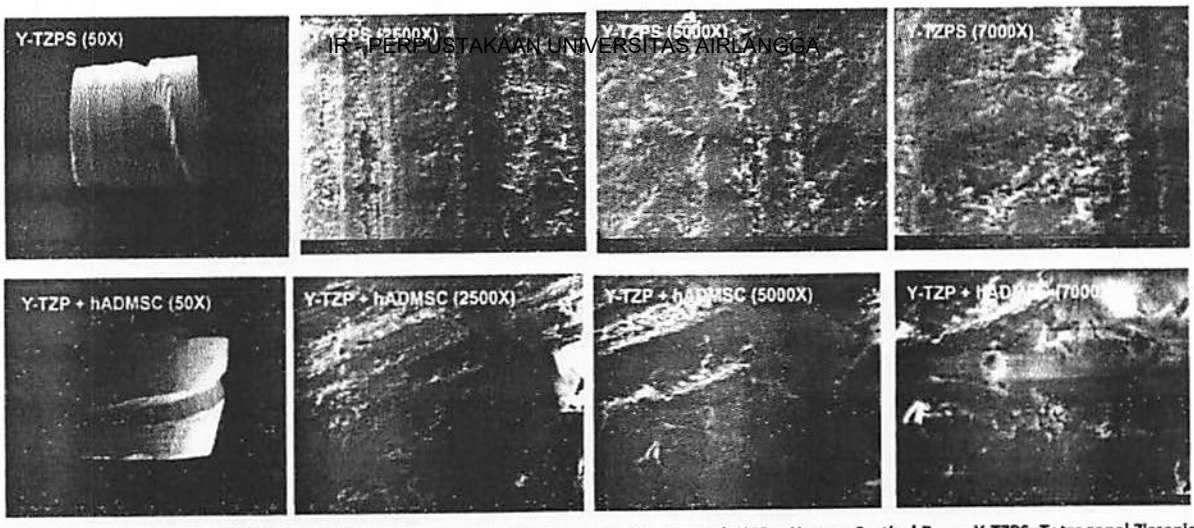

Figure 4. Toxicity Test TT of Y-TZPSs and HCB (Before and After MTT Assay). CC = Control, HCB = Human Cortical Bone, Y-TZPS=Tetragonal Zirconia

Polycrystal

Test Method	Specification	Result ADSC-HMN-P4-220518
Viability	FIO	94.87 %
Biomarker CD 1052	NLT 95 %	97.41 %
Biomarker CD 732	NLT 95 %	99.56 %
Biomarker CD 902	NLT 95 %	97.61 %
Biomarker CD 452	NMT 2 %	1.42 %
Biomarker CD 342	NMT 2 %	1.45 %
Biomarker CD 142	NMT 2 %	0.74 %
Biomarker CD 192	NMT 2 %	0.83 %
Biomarker HLA-DR	NMT 2 %	0.84 %

Table 1. Characterization Analysis (CA) of hAD MSCs. Cell NMT, Cells, Remark: FIO: For Information Only, NLT: count: Not More Than 647,500 Lower Than,

No	Medium Control (MC)	CC	HCB	Y-TZPS
1	0,095	0,942	0,843	0,910
2	0,092	0,945	0,849	0,923
3	0,091	0,943	0,852	0,921
4	0,092	0,949	0,846	0,920

5	0,092	0,943	0,850	0,918
Total	0,462	4,722	4,240	4,592
Mean	0,092	0,944	0,848	0,918
(%) Living Cells (LC)			89	97

Table. 2 Results of the MTT Assay on Scaffolds

The other side, effect of honey on fracture defect was only shown the difference without remarkable (Sahin, et al., 2018). In detail the result of this study was based on, ALP serum as follows,

Tabel-3: Analysis of Average ALP Serum

Treatment	X ± SD
Control Group	270.00 ± 31.22^{a}
1st Honey Group	536.67 ± 107.74^{b}
2nd Honey Group	533.33 ± 55.07^{b}
3rd Honey Group	478.33 ± 76.37^{b}

The different superscript alphabet in the same column indicate very significantly different ($P<0.05$)

The average ± SD of ALP serum concentration in control group are 270 ± 31.22 that the lowest level than Honey Group. The average of ALP serum concentration Honey Group above 400 IU/L. The normal ALP mean of male Wistar rat was 238 IU/L in 149-328 IU/L interval (Charles River Laboratory, 1998).

Achievement Of Output:

1. One of this manuscript already published in Journal of Acta Informatica Medica, a journal with Scopus indexing. Q3 on November 2018
2. One of member team research already presenting in International Endodontic meeting
3. One of member team research also attend the international meeting as a participant

Chapter 6.
Planning research 2nd year

The highlight of next step to determine increasing capacity of honey supplementation has a beneficial effect on fracture healing by supporting the osteoblastogenesis on Y-TZPSs-hADMSCs. Pro-osteoblastic effect of honey could be revealed by ALP serum increasement which indicates proliferation and maturation of osteoblast.

Chapter 7

Conclusion and suggestion

This study conclude that Y-TZPSs- hADMSCs as a biomaterial had high biocompatibility for osseointgrated acceleration of implantation.

Suggestion: for increasing capacity of Y-TZPSs- hADMSCs using Honey supplementation has a beneficial effect on fracture healing by supporting the osteoblastogenesis. Pro-osteoblastic effect of honey could be revealed by ALP serum increasement which indicates proliferation and maturation of osteoblast.

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ORIGINAL PAPER

Biocompatibility of Yttria-Tetragonal Zirconia Polycrystal Seeded with Human Adipose Derived Mesenchymal Stem Cell

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ABSTRACT

Introduction: The scaffold is a place for regeneration of new bone and bone tissue growths in tissue engineering applications. hADMSC is a multipotent cell which can differentiate into osteogenic, chondrogenic and adipogenic. Y-TZP has been shown to have several advantages over other ceramics because of its hard nature, namely fracture toughness and high flexural strength. **Aim:** This study aimed to analyze the biocompatibility of Y-TZP as a scaffold seeded with hADMSCs by in vitro analysis. **Material and Methods:** This research involved several processes, namely Y-TZPS manufacture process, XRD examination, differentiation and characterization of hADMSC, SEM observation, and then TT. **Results:** The results of the XRD examination showed that Y-TZPSs had sharp peaks. It suggests that they had high crystal purity. The marked expression of the characterization of hADMSC is the positive expression of Cluster of differentiation (CD), namely CD 90, CD 73 and CD 105 above NMT and negative expressions of CD 14, CD 19, CD 34, CD 45 and also HLA-DR below NLT. The analysis of observations on the Y-TZPSs with SEM, subsequently, indicated the porosity of Y-TZPSs, as a result, the adhesion of HADMSCs occurred and grew in the porosity in the Y-TZPSs. **Conclusions:** Y-TZPSs with low porosity and toxicity can be able to proliferate and differentiate if seeded with hADMSC. Y-TZPSs are expected to be used as implantable biomaterials using hADMSCs with high biocompatibility.

Keywords: Electron Scanning Microscopy, Mesenchymal Stem Cell, Toxicity, X-ray Diffractions, Y-TZP ceramic

1. INTRODUCTION

Tissue engineering has components consisting of cells, scaffolds, growth factors called triad of network engineering. The scaffold is a material made from polymeric material that can add material support to the material in the attachment of the cell and the development of subcutaneous tissue. The scaffold is also known as an important component in tissue engineering (1). The scaffold is a place to regenerate cell growth and new bone tissue in tissue engineering applications (2).

On the other hand, titanium implants often trigger periimplanitis and mucositis periimplanitis elevating from 28% to 56% among 80% of subjects taken (3). Hence, implants made of Zirconia can be suggested to patients who have an allergic reaction to titanium or other metals. In the biological environment, test and osseointegration power, there

Tetragonal Zirconia Polycrystal (Y-TZP), consequently, can be considered as an alternative dental implant material that can replace implant material that has been used, namely Titanium (5). Y-TZP is also known to be more esthetically and bio compatibly than titanium, thus, Y-TZP as an alternative dental implant material can function properly as a scaffold (6, 7). Besides, excess Y-TZP is rigid and not easily broken compared to other ceramic materials. In the field of dentistry, ceramic materials have high biocompatibility, namely thermal conductivity, low corrosion rates and high aesthetics in mucogingival in implant preparation (8).

In addition, Mesenchymal stem cells (MSC) can play an active role in repairing and maintaining tissue homeostasis. Osteoblasts can have potential for bone regeneration where the ability of cells that differentiate as effector cells

is a multipotent cell which can differentiate into osteogenic, chondrogenic, and adipogenic. hADMSC has many similar characteristics of Bone Marrow stem cells (BMSCs) but hADMSC has a high proliferation rate compared to BMSCs (10).

2. AIM

This research aimed to calcify the biocompatibility of Y-TZP as a scaffold (Y-TZPS) seeded with hADMSCs by performing X-Ray Diffraction (XRD) examination, scanning electron microscope (SEM) observation test, and in vitro toxicity test (TT).

3. MATERIAL AND METHODS

Y-TZPS Manufacturing Process

In this research, scaffolds used were made in a dental laboratory. Scaffold samples used were taken from Y-TZP [Vita YZ Interne Untersuchungen, VITA F & E: VITA Zahnfabrik H. Rauter GmbH & Co. KG Ressort Forschung und Entwicklung Spitalgasse 379713 Bad Säckingen Dipl.-Ing] [11]. Y-TZP have grooves and holes in their rod section with a Y-TZP size (Ø = 2.9mm, P = 3mm). In the manufacturing of Y-TZPS, sterilization was carried out using gamma cell radiation at Irradiation, Electromechanics, and PAIR-BATAN Instrumentation Center in Jakarta.

XRD Examination

Samples of Y-TZP were made in the forms of fine crystalline powder with a minimum size of 5 mg. To conduct XRD examination, [XRD D8 Focus, The Bruker Corporation (NASDAQ: BRKR), Billerica, Massachusetts, USA] and HCB were taken from male resection maxilla patients who were trafficking victims. Crystal powder that had been pressed into sample container, had a smooth surface, and was resistant to the samples at a 45-degree angle. A small amount of solid sample by depositing the substrate on a thin film placed on a sliding glass from the microscope, then the intensity of the beam is reduced from 40 KV and 40mA to 30kV and 30mA.

Log XRD analysis

The log must be filled in to operate the XRD before starting the test to be recorded. The XRD operation aims to check the alarm lights on the right side of the. Next, the XRD spreadsheet log on the desktop was filled in. KV setting, then was conducted by increasing the addition of 10kV at 30 seconds to 40kV. Subsequently, <set> button was pressed after every change. An mA setting of 5-10 then was carried out with an addition of 10mA to 40mA. Afterwards, a permanent parameter file was created for automatic scanning stored in the XRD wizard.

Adipose Sampling Procedure

Adipose or body fat tissue samples taken from participants with elective indications and no medical complications at Universitas Airlangga Hospital, Surabaya in accordance with certain criteria, such as pregnant women who underwent the birth process by cesarean section or Cesarean section due to abnormal fetus location

Adipose sampling was performed with an oval slice on the upper surface, remaining adipose on the inner surface to-

and taken to the Research and Development of Stem Cell Universitas Airlangga Laboratory (RDSCUAL), Surabaya for hADMSC isolation, cultural expansion, and TT.

Those Adiposa were washed with PBS solution, those adipose was cut and chopped until smooth and then were added by collagenase enzyme. Afterwards, they were soaked and incubated at 37°C for 45 minutes. It was added to the pellets, and planted on a 10 cm plate until the cells were attached to the base of each petri and labeled with the patient's identity in the form of the name and processing date, and then incubated in CO_2 incubator.

Isolation and Culture Expansion Procedure of hADMSC

Isolation of hADMSC was using lipoaspirate enzymatic to separate SVF. Cells, on the other hand, are known consisting of stroma, progenitor SCs, WBCs, ECs, periocytes, and RBCs. Isolation and Culture Expansion Procedure of hADMSC according to (Banyard et al., 2015) without modification until passage 4 (12).

Characterization and Differentiation of hADMSC Phenotype

Characterization and differentiation of the MSC phenotype in hADMSC culture were conducted in two types of immunostaining methods, namely immunocytochemical staining and flowcytometry test (FT). Identification of hADMSCs was carried out at the SCIL of PT. Kalbe Farma Tbk with MSC phenotype kit after the fourth passage. Cells that have adipogenic, osteogenic, or chondrogenic properties are oil red-O staining (OROS) for adipogenic and Alizharin staining for osteogenic by dripping lipids and then fixed with 4% formalin (13). Differentiation of hADMSC to determine adipogenic and osteogenic according to (Jeon et al. 2016) without modification (20).

Flowcytometry

Flowcytometry was performed to make monolayer cells change into single cells through trypsination process. After that, Flowcytometry is carried out according to the procedure from (Van Pham et al, 2016) without modification (14, 20, 21).

hADMSC Seeding Procedure on Y-TZPS

In Y-TZPS, hADMSCs were hatched by immersing them in DMEM / F12 medium for 1 day. The old medium, then was removed and replaced by the new one. After one day, the Y-TZPSs were put into 24 culture wells (M24) with 2 x 10^6 cells (200 µL/well). Those wells, then were incubated for 1 hour at 37° C with 5% CO_2. In the well the medium was added as much as 1.3 mL / well and incubated with 5% CO_2 at 37° C. Periodically, the tubes were rocked to mix cells with the suspensions and Y-TZPSs. The cell-coated Y-TZPSs then were ready in the next 3 days for SEM.

Observation of Y-TZPS Seeded with hADMSCs

Cells that had been cultured into the Y-TZPSs were fixed with 2% glutaraldehyde solution for 2 hours at 40° C, then washed with PBS solution 3 times for 5 minutes, replaced with 1% OAS for 1 hour at 40° C, and washed with PBS again. After that, they were dehydrated with alcohols from low to absolute levels for 15 minutes each, then replaced with absolute AAL as a preservative until dry time. Next, Y-TZPS

evaporator fixation tool. The Y-TZPSs then were ready to be observed and photographed with SEM at the DME, FIE, ITS, Surabaya.

TT on Y-TZPS and HCB

TT on Y-TZPS and HCB against hADMSC cell culture, trypination of a petri plate containing 2.5 x 10^6 cells was carried out. Next, resuspension was conducted in DMEM / F12 medium and centrifuged. Afterwards, the Pellets are planted into M96 of 5 x 10^4 cells / well, then incubated with 5% CO_2 at 37° C for 24 hours. When 80% of the growth was obtained, the Y-TZPS were added to ½ parts of the wells. Subsequently, 100 µL of DMEM / F12 medium was added, and incubated with 5% CO_2 at 37° C for 20 hours. After that, the wells were put in MTT as much as 5 mg / mL (25µL / well), then incubated for 4 hours, and observed under an inverted microscope. The Y-TZP and medium, then were discarded, and sDMSO was added into each well as many as 200 µL. Color changes in those wells were then read by the MTT Assay with Elisa Reader at a wavelength of 595 nm.

Ethics

This study has been evaluated and approved by RSUA (Airlangga University Hospital) Ethics Committee, Surabaya Indonesia with Ethical Clearance Number: 107/KEH/2018.

4. RESULTS

The manufacturing of Y-TZPSs and mineral analysis

In the manufacturing of Y-TZPSs, there was ad distortion so that they did not only experience shrinkage in both dimension and density of their material, but their color also changed into browner than their original white color. These changes, as a result, had an impact on both the material density and the Y-TZPS density (Figure 1A). To know porosity in the scaffolds, magnification in using an SEM was required.

The results of the mineral analysis with XRD examination were illustrated by a graph of Y-TZP, HCB, and a combination of both (Figure 1B). In Y-TZP, there was a high and sharp peak on the graph of material analysis with XRD examination. The Y-TZP graph was also known to have a low frequency. Similarly, in HCB there was a sharp peak with low frequency. Thus, to see their differences, XRD examination was performed on Y-TZP minerals combined with HCB minerals. The results showed that the height of the peak in Y-TZP was higher than that in HCB. Coefficient of thermal expansion had been determined by XRD measurement (15).

Characterization and differentiation of hADMSC phenotype

Isolation and Culture Expansion on hAMDSC of passage 1 was observed on the 3rd day followed by passages 2, 3, and 4 respectively until day 12. In passage 4, the cell density increased and was ready for both the adipogenic and osteogenic differentiations (AD and OD) of hADMSCs and the characterization of hADMSCs (Figure 2).

Based on results of the ADA of hADMSC with OROS, there were more vacuoles than in cell controls. This shows that hADMSCs have properties that can be used for AD.

Whereas the osteogenic differentiation analysis of hADMSCs with alizharin staining, it is indicated that hADMSCs have properties that can be differentiated towards

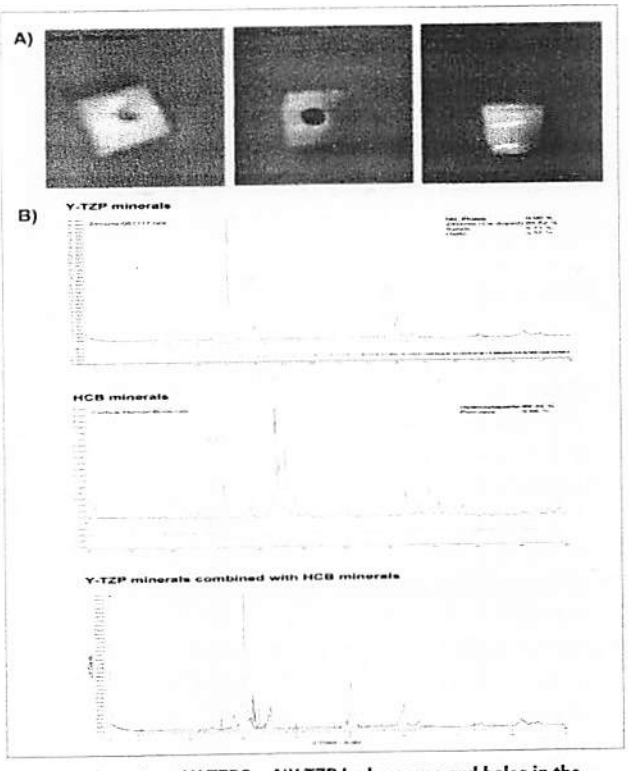

Figure 1. Distortion of Y-TZPSs. A)Y-TZP had grooves and holes in the rod section with size (Ø = 2.9mm, P = 3mm). B) mineral analysis with XRD examination. The peak on the graph of material analysis with XRD examination of Y-TZP minerals, HCB minerals, and Y-TZP minerals combined with HCB minerals

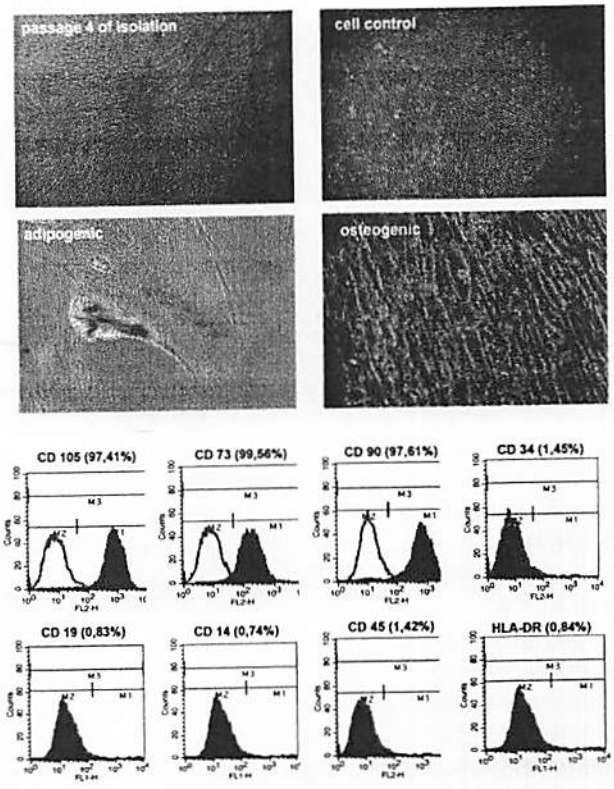

Figure 2. Isolation, differentiation, characterization and Graph Characterization Analysis (CA) of hADMSC. Adipogenic differentiation analysis (ADA) of hADMSCs with oil red-O staining (OROS). Osteogenic differentiation analysis (ODA) of hADMSCs with Alizahrin Staining. CA by immunocytochemical staining and flowcytometry test (FT).

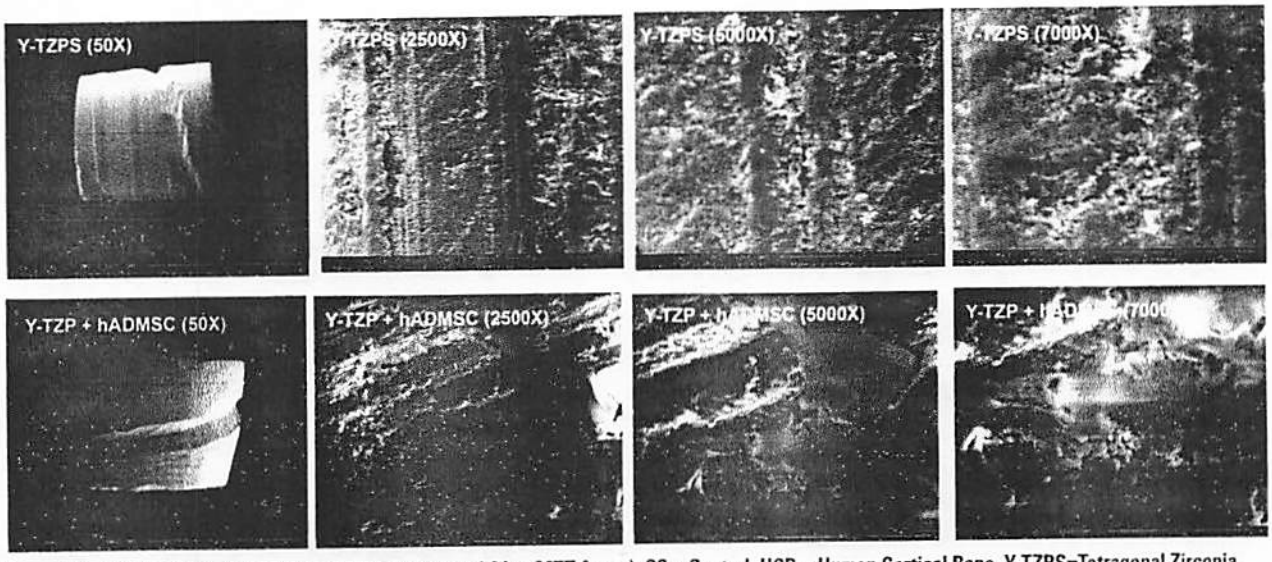

Figure 4. Toxicity Test TT of Y-TZPSs and HCB (Before and After MTT Assay). CC = Control, HCB = Human Cortical Bone, Y-TZPS=Tetragonal Zirconia Polycrystal

Test Method	Specification	Result ADSC-HMN-P4-220518
Viability	FIO	94.87%
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Biomarker CD 732	NLT 95 %	99.56 %
Biomarker CD 902	NLT 95 %	97.61 %
Biomarker CD 452	NMT 2 %	1.42 %
Biomarker CD 342	NMT 2 %	1.45 %
Biomarker CD 142	NMT 2 %	0.74 %
Biomarker CD 192	NMT 2 %	0.83 %
Biomarker HLA-DR	NMT 2 %	0.84 %

Table 1. Characterization Analysis (CA) of hADMSCs. Cell count: 647,500 Cells, Remark: FIO: For Information Only, NLT: Not Lower Than, NMT, Not More Than

No	Medium Control (MC)	CC	HCB	Y-TZPS
1	0,095	0,942	0,843	0,910
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4	0,092	0,949	0,846	0,920
5	0,092	0,943	0,850	0,918
Total	0,462	4,722	4,240	4,592
Mean	0,092	0,944	0,848	0,918
(%) Living Cells (LC)			89	97

Table. 2 Results of the MTT Assay on Scaffolds

34 (1.45%), CD 45 (1.42%) and also HLA-DR (0.84%) were below 2%.

Observation Analysis (OA) on Y-TZPS with SEM

Results of OA on Y-TZPS using SEM with magnifications of 50x, 2500x, 5000x, and 7000x showed porosity in Y-TZPS. Meanwhile, results of the OA on Y-TZPS seeded with hADMSCs using SEM showed that there were cells attached to the surface of Y-TZPS seeded and fixed (Figure 4).

Toxicity Test

Results of the TT on Y-TZPSs and HCB in 96M revealed that both Y-TZPSs and HCB were not toxic to hADMSC.

Table 2 showed that Y-TZPSs seeded with hADMSCs had a higher percentage of LCs is 97% compared to HCB with

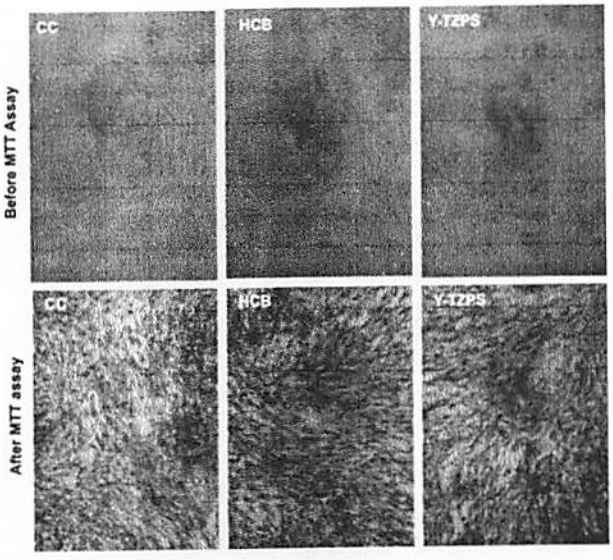

Figure 3. Observation Analysis on Y-TZPS by Scanning Electron Microscope (SEM). Y-TZPS 50x, 2500x, 5000x, and 7000x, (f) Y-TZPS seeded with hADMSCs 50x, 350x, 2500x, 5000x and 7000x.

and Y-TZPSs both before and after MTT Assay.

5. DISCUSSION

The results of the XRD examination showed that Y-TZPSs had a sharper peak than human bone. This indicates that minerals contained in the Y-TZPSs have higher crystalline purity than HCB. More ever, the characterization and differentiation of adipose phenotypes taken from selected human donors show that MSC of the adipose referred to as hADMSC. Expressions emerged in the differentiation of hADMSCs then were used for AD and OD. The expressions marked in the characterizations that are not only positive are from CD 90, CD 73 and CD 105 above 95%, while they are on CD 14, CD 19, CD 34, CD 45 and also HLA-DR below 2% which means that cells as MSC.

Observation of Y-TZPs with SEM, furthermore, found that there were many large porouses due to the porosity of Y-TZPSs after the 7000x enlargement, which HADMSCs

icity between the Y-TZPS and the HCB. hADMSCs that had been seeded could also live and would not be toxic to the Y-TZPS. Even, they had more LCs than HCB as depicted in Table 2 and Figure 4. As a result, biomaterials are developed to interact with the tissue, so they can induce repairing of the tissue (16).

Y-TZPSs, has a great aesthetical performance, biocompatibility, and mechanical properties (5). HCB has been reported to be effective to induce regeneration in periodontal intra-coniac deficiency (17). However, the HCB is isotropic and not homogeneous. The osseointegration rate is 100%, also shown to be incompatible with a non-linear frictional contact analysis and good osseointegration between implants and HCB (18). Based on the data above, Y-TZPSs have high porosity, but hADMSCs are still able to adhere and grow, as well as not lead to toxicity. The hypothesis with Y-TZPSs-hADMSCs in tissue engineering so that it has large osseointegration capabilities if Y-TZPSs are without hADMSCs. High power and osseointegration acceleration can reduce implantation failure. In the future, Y-TZPSs-hADMSCs made a tissue engineering in dental implantation and orthopedics.

6. CONCLUSION

This study conclude that Y-TZPSs- hADMSCs as an biomaterial had high biocompatibility for osseointgrated acceleration of implantation.

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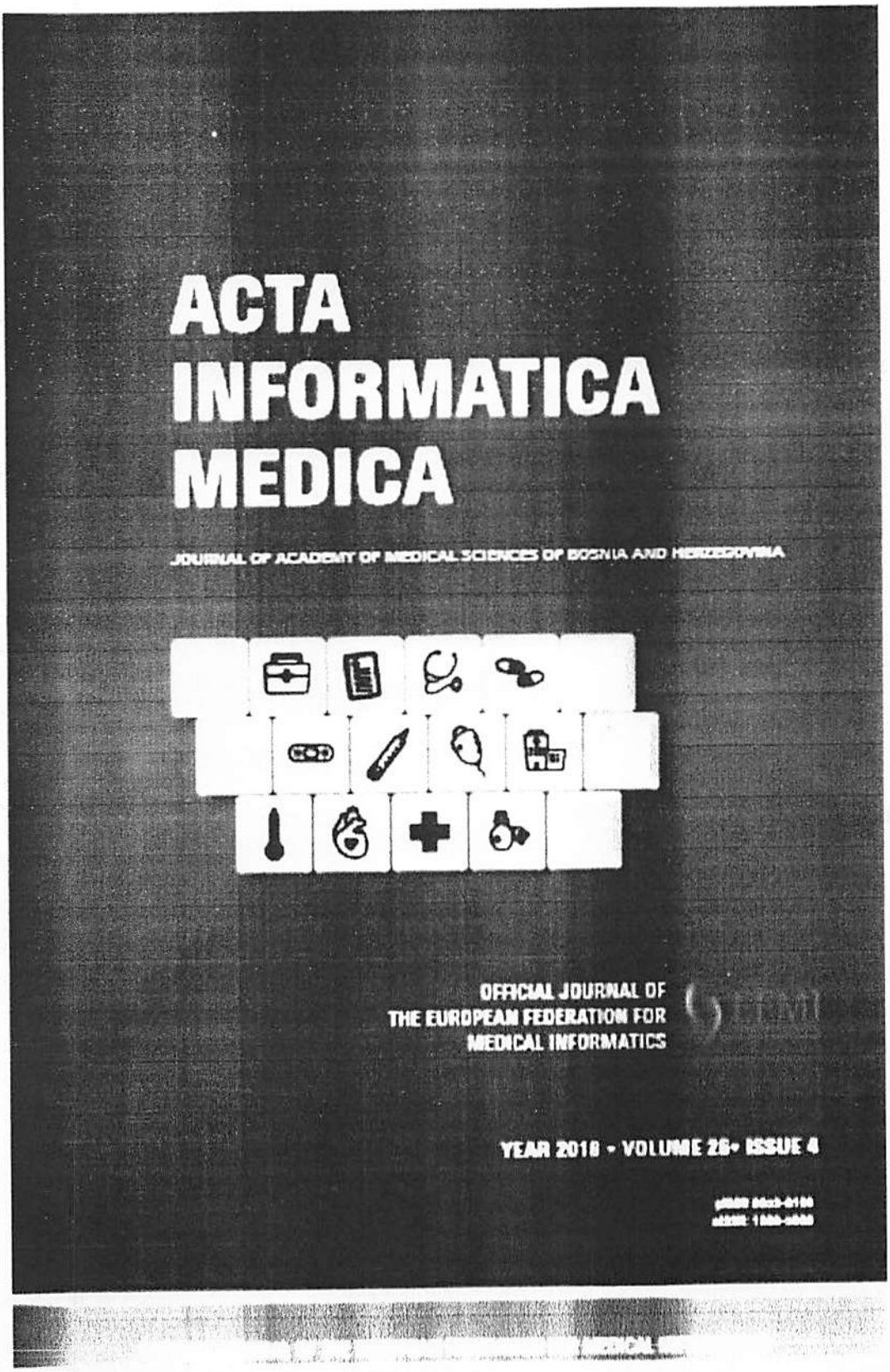
Lampiran 2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